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REPORT DOCUMENTATION PAGE

Form Approved OMB NO. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	3/16/98	Final 4/15/96 - 9/30/97

4. TITLE AND SUBTITLE

Characterization of asgC a gene required for cell-cell signaling during early development of Myxococcus xanthus

5. FUNDING NUMBERS

Grant DAAH04-96-1-0063

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8. PERFORMING ORGANIZATION REPORT NUMBER

RF No 9400491RB1

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

ARO 33956.3-LS

11. SUPPLEMENTARY NOTES

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

13. ABSTRACT *(Maximum 200 words)*

Myxococcus xanthus is a soil bacterium that forms spore-filled fruiting bodies in response to nutrient limitation coupled with high cell density. Extracellular A-signal, which is composed of amino acids and peptides, is produced during early development and is thought to function as a cell density signal. The *asgC* gene is one of three known regulatory genes that are required for A-signal production. The *asgA* and *asgB* genes encode a signaling kinase and a putative transcription factor, respectively. The longterm goal of this project was to understand the role of *asgC* in A-signal production. To this end, the DNA sequence of *asgC* and the only known *asgC* mutant allele, *asgC767*, was determined. The asgC gene encodes RpoD, the major sigma factor in *M. xanthus*. The second aim was to isolate pseudorevertants of *asgC767* that have regained the ability to produce fruiting bodies and extracellular A-signal. Approximately 40 independent pseudorevertants have been isolated, and their characterization has been initiated. Analysis of these mutants may lead to the identification of additional components of the signal transduction pathway leading to A-signal production.

14. SUBJECT TERMS	15. NUMBER IF PAGES
cell-cell signaling, cell density sensing, development sporulation, sigma factor, phosphorelay, Myxococcus xanthus	9
	16. PRICE CODE

17. SECURITY CLASSIFICATION OR REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

(1) AND (2): OMIT

(3) LIST OF APPENDIXES:

Appendix A:

Davis, John M., Jocelyne Mayor, and Lynda Plamann. 1995. A missense mutation in *rpoD* results in an A-signaling defect in *Myxococcus xanthus*. Molecular Microbiology **18**:943-952.

Appendix B:

Plamann, Lynda and Heidi B. Kaplan. 1998. Cell-density sensing during early development in *Myxococcus xanthus*.. *In* G. Dunney and S. Winans (ed.), Cell-cell communication in bacteria. American Society for Microbiology, Washington, DC.

(4) STATEMENT OF THE PROBLEM STUDIED:

Myxobacteria are unique prokaryotes in that they display a range of multicellular behaviors requiring cell-cell interactions (for review, see (Shimkets, 1990)). Perhaps the most impressive social behavior of the myxobacterium *Myxococcus xanthus* is the building of multicellular, haystack-shaped structures known as fruiting bodies. Upon starvation, many thousands of cells glide to aggregation centers where they participate in the formation of a fruiting body. Cells within the nascent fruiting body differentiate into ovoid, heat- and desiccation-resistant myxospores. The long-term goal of this research is to determine how individual cells sense starvation and regulate production of an extracellular signal (A-signal) required during the initiation of multicellular development.

The *asg* mutants

The *asg* (A-signal generating) mutants of *M. xanthus* fail to generate extracellular A-signal and are arrested early in development. As a postdoctoral fellow in Dr. Dale Kaiser's laboratory, I carried out biochemical studies to purify and characterize A-signal. My colleagues and I showed that A-signal is composed of amino acids and peptides, and it is generated by extracellular proteases (Kuspa et al., 1992, Plamann et al., 1992). A high cell density is required for fruiting body formation, and we propose that sensing of extracellular amino acids provides a way for Myxococcus to determine the cell density of the starving population of cells (Kuspa et al., 1992).

There are three known *asg* loci: *asgA*, *asgB*, and *asgC* (Kuspa and Kaiser, 1989). The overall goal of the work in my current laboratory is to understand the roles of the *asg* genes in A-signal production and starvation sensing. The *asgA* and *asgB* genes have been studied as part of another project. A summary of these studies is given below, and provides the context for evaluation of the work described in this final progress report. **The**

purpose of this project was to characterize *asgC* as well as uncover additional components of the signal transduction pathway(s) required for sensing of starvation and A-signal production in *M. xanthus*.

asgA

The deduced amino acid sequence of AsgA was found to have a remarkable similarity to members of the histidine protein kinase family and the response regulator family of the so-called "two-component" regulatory systems. In most two-component systems, the histidine protein kinase domain or "transmitter" is part of a transmembrane sensor protein, and the response regulator domain or "receiver" is part of a transcriptional regulator protein. When the sensor is stimulated by ligand binding, it is autophosphorylated by its histidine protein kinase domain. The phosphoryl group is transferred to the receiver domain of the transcriptional regulator; phosphorylation modulates the activity of the DNA binding domain and ultimately results in a change in gene expression (Parkinson and Kofoid, 1992) .

In most of the two-component signal transduction systems, the transmitter and receiver domains are located on separate polypeptides (Parkinson and Kofoid, 1992). AsgA is unusual because it contains both domains; it is unique because it is the only protein we know of that has the receiver at the N-terminus and the transmitter at the C-terminus. In addition, AsgA appears to lack the hydrophobic, membrane-spanning regions that characterize most of the sensors, and, therefore, is likely to function in the cytoplasm. Because AsgA appears to lack "input" and "output" domains (that is, domains that interact with a signaling ligand or bind to DNA), it is likely to interact with other signaling proteins that have input or output functions. AsgA may function in the middle of a phosphorelay similar to the phosphorelay that controls initiation of sporulation in *Bacillus subtilis* (Burbulys et al., 1991, Grossman, 1991)

asgB

A clue to the function of *asgB* was provided by an examination of its DNA sequence. The deduced 163 amino acid sequence of AsgB contains a region similar to conserved region four of sigma factors (Plamann et al., 1994). Region four is characterized by its location at the C-terminus and a conserved helix-turn-helix structure that directly contacts the -35 region of promoter sequences (Dombroski et al., 1992, Gardella et al., 1989, Lonetto et al., 1992, Siegele et al., 1989). There is no evidence for the presence within AsgB of region two, a second highly conserved region that is believed to interact with core polymerase and the -10 regions of promoters; therefore, it is unlikely that AsgB is a sigma factor. Instead, AsgB may function as a transcriptional activator or repressor.

(5) SUMMARY OF THE MOST IMPORTANT RESULTS

DNA sequence analysis of *asgC*

We cloned *asgC* and localized the gene to a region of the *M. xanthus* chromosome that contains genes homologous to those of the *E. coli* macromolecular synthesis (MMS) operon. The MMS operon contains genes that are necessary for the initiation of translation (*rpsU*), DNA replication (*dnaG*), and RNA synthesis (*rpoD*). The first gene of the operon, *rpsU*, encodes the ribosomal protein S21, which interacts with the 16S ribosomal RNA sequences complementary to the ribosome-binding sites of mRNAs. Initiation of DNA replication requires primase, the product of *dnaG*, the second gene of the MMS operon. Primase is responsible for synthesis of RNA primers that are required for the production of lagging-DNA strand Okazaki fragments (Lupski and Godson, 1984, Versalovic et al., 1993). The *rpoD* gene encodes the major sigma factor, which is responsible for promoter sequence recognition during initiation of mRNA synthesis. A nested set of deletion plasmids derived from a clone containing the region homologous to the MMS operon was constructed using exonuclease III. By analyzing the ability of these plasmids to rescue development of the *asgC* mutant, we localized the *asgC767* mutation to within the *rpoD* gene. We cloned *asgC767*, the only known *asgC* mutant allele, and found that it contains two adjacent transition mutations (G to A), resulting in a glutamate to lysine substitution at position 598 of RpoD. This substitution lies within region 3.1, which contains many acidic residues and is conserved among sigma-70-like sigma factors (Lonetto et al., 1992).

The *asgC767* (*rpoDEK598*)) mutant does not appear to have a general defect in growth, and its vegetative phenotypes (tan rather than yellow colony color, decreased cohesiveness, and decreased extracellular enzyme production) are very similar to those of the *asgA* and *asgB* mutants. We found that, as in *asgA* and *asgB* mutants, expression of Ω4521 is reduced during growth and development in the *asgC767* background. Furthermore, expression of Ω4521 in the *asgC* mutant is restored upon addition of A-signal. These observations are consistent with the hypothesis that the *rpoD(EK598)* mutant has a defect in A-signaling that is similar to the defect of the *asgA* and *asgB* mutants, rather than a more general defect in transcription initiation.

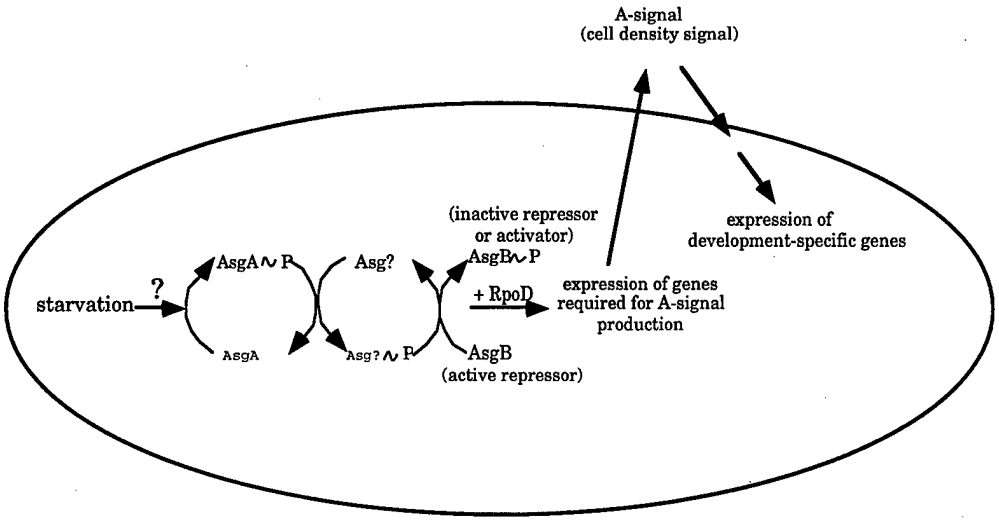
Model for the role of AsgC (RpoD) during development

One hypothetical explanation for the A-signaling defect observed in the *rpoD(EK598)* strain is that the mutant sigma subunit fails to productively interact with a transcriptional regulator that affects A-signal production. Mutations that affect interactions between RNA polymerase and transcription factors have been identified in the genes encoding the alpha and sigma subunits of RNA polymerase (Ishihama, 1993). *E. coli rpoD* mutations that affect interactions with the transcription factors PhoB (Makino et al., 1993), CRP (at the P1*gal* promoter) (Kolb et al., 1993) AraC (Hu and Gross, 1985), and the λ cI repressor (Kuldell and Hochschild, 1994, Li et al., 1994) have been localized to region 4. Kumar et al. (Kumar et al., 1994) suggest,

from data provided through a deletional analysis of the C-terminal portions of *rpoD*, that a region extending from at least region 3.2 to upstream of region 4.2 may be involved in association with transcription factors. More recently, Bramucci et al. (Bramucci et al., 1995) identified a mutation in a *B. subtilis* sigma factor gene (*spo0H*) that suppresses the transcriptional defects of a mutant form of the transcription factor Spo0A (Spo0A9V). This *spo0H* mutation is located between the sequences encoding regions 2 and 3 and is proposed to allow SpoOA9V to interact with the mutant sigma-H, restoring transcriptional activation. Similarly, the A-signaling defect observed in the *rpoD(EK598)* strain may be caused by a failure of the mutant sigma subunit to functionally interact with a transcriptional regulator necessary for A-signal production. If this hypothesis is correct, the isolation and characterization of suppressors of *rpoD(EK598)* may allow identification of the hypothetical transcriptional regulator or its target genes. An alternative hypothesis is that the mutant sigma factor has a greater affinity for core polymerase, preventing alternative sigma factors necessary for A-signal production from gaining access to core. In this case, a suppressor analysis may result in the identification of genes encoding alternative sigma factors or RNA polymerase mutants that have altered affinities for sigma subunit.

A model for the A-signal-generating pathway

The figure below illustrates one of many models for the role of the *asg* gene products in A-signal generation. Given that the *asg* mutants have very similar phenotypes and the *asg* genes encode proteins that appear to have regulatory functions, we hypothesize that the *asg* gene products function together in a regulatory pathway that is required for production of extracellular A-signal. An alternative model is one in which the *asg* gene products function in different, but related, regulatory pathways. In the figure, AsgA (a histidine protein kinase) is shown interacting with an unknown starvation sensor, which may be another histidine protein kinase, a serine/threonine kinase, or a small-molecule phosphodonor. This interaction results in activation of the AsgA autokinase, and AsgA is converted to AsgA-phosphate. The phosphoryl group is then transferred to an unidentified protein or through a series of proteins, and finally to AsgB (a putative transcription factor). In this model, AsgB acts as a repressor of early developmental genes when it is nonphosphorylated and as an activator of these genes when phosphorylated. Expression of the genes required for A-signal production depends upon wild type sigma factor, perhaps for interaction with phosphorylated AsgB and/or ppGpp. Expression of these genes, which may include protease genes, results in extracellular A-signal production. Finally, sensing of A-signal leads to the expression of development-specific genes such as Ω4521. It is easy to imagine several variations of this model. For example, the regulator at the end of the pathway may be a transcriptional activator whose production is regulated by AsgB, or AsgA may function downstream rather than upstream of AsgB.



A model for the A-signal-generating pathway (see text).

Isolation and characterization of pseudorevertants of the *asgC767* mutant

The isolation and characterization of pseudorevertants is a powerful genetic approach that will provide insight into the A-signal generating pathway. Secondary mutations that reverse the mutant phenotype, in this case, the inability to form fruiting bodies and sporulate, are known as "suppressor" mutations (Botstein and Maurer, 1982). With the exceptions of informational suppression and back mutation, the manner by which the mutant phenotype is reversed can tell us much about the function of the mutant gene. Bypass suppressors, which are gene-specific but not allele-specific, act on null mutations as well as missense mutations because the original mutant protein is not required for suppression (Parkinson, 1995). For example, bypass of the *asgC* defect may occur if the cell acquires a mutation that obviates the need for A-signal to turn on A-signal dependent genes during development. In short, some bypass suppressors will identify downstream components of the A-signal-generating pathway, whereas others are likely to provide information about how the pathway operates without identifying pathway components.

Conformational suppressors reverse the mutant phenotype by restoring activity of the mutant protein. Activity may be restored following a change at a secondary site within the mutant protein that alters its folding, stability, or function. Of greater interest with respect to this study, activity may be restored by a compensating change in a protein that interacts with the original mutant protein. Isolation of this type of interaction suppressor, which is highly allele-specific, requires the presence of stable mutant protein. Interaction suppressors of *asgC* would identify components of the A-signal generating pathway that lie immediately upstream or downstream of AsgC.

We have isolated *asgC* pseudorevertants that form fruiting bodies and sporulate. A protocol described by Rhie and Shimkets (Rhie and Shimkets, 1989) was used. In brief, *asgC* mutant cells were UV-mutagenized, and plated on clone-fruiting (CF) medium, a medium that supports development of wild type cells but not *asg* mutants. The plates were incubated for 3 to 5 days at 32°C, and then were heat-treated to kill vegetative cells. Heat-treated cells were resuspended in buffer, subjected to sonication (to disrupt any remaining vegetative cells and break up clumps of spores), and plated on a rich medium. Colonies were then tested for fruiting body formation on starvation plates and/or CF medium. Only one pseudorevertant/mutagenesis was saved for further analyses, unless pseudorevertants with obviously different phenotypes were observed.

We have isolated approximately 100 *asgC* pseudorevertants from 39 independent selections. We have found that about 2/3 of our *asgC* suppressors are linked to *asgC*. These suppressor mutations are most likely intragenic suppressor mutations or reversions. For these studies, we have concentrated on those pseudorevertants that contain suppressor mutations that are unlinked to *asgC*.

Analysis of A-signal production by the pseudorevertants

We have used the standard A-signal bioassay to determine whether the pseudorevertants have restored A-signal production. So far, we have determined that there are at least three classes of pseudorevertants with respect to A-signal production: One class produces wild type levels of A-signal, a second class produces no A-signal (a level comparable to that of the original *asgC767* mutant), and a third class produces an intermediate level of A-signal. The mutants that produce little or no A-signal appear to have bypassed the developmental requirement for A-signal.

(6) LIST OF ALL PUBLICATIONS AND TECHNICAL REPORTS

The initial proposal for this research was submitted to the ARO in August, 1994. However, funding was delayed until 15 April, 1996. The following manuscript resulted from the work that was carried out during the intervening period (see Appendix A).

Davis, John M., Jocelyne Mayor, and Lynda Plamann. 1995. A missense mutation in *rpoD* results in an A-signaling defect in *Myxococcus xanthus*. Molecular Microbiology **18**:943-952.

The following invited book chapter is in press (see Appendix B):

Plamann, Lynda and Heidi B. Kaplan. 1998. Cell-density sensing during early development in *Myxococcus xanthus*.. *In* G. Dunney and S. Winans (ed.), Cell-cell communication in bacteria. American Society for Microbiology, Washington, DC.

(7) LIST OF ALL PARTICIPATING SCIENTIFIC PERSONNEL

Lynda Plamann, Ph.D. (P.I.)
John. M. Davis (graduate student)
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(8) REPORT OF INVENTIONS

none

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(10) APPENDIXES

Appendix A

Molecular Microbiology (1995) **18**(5), 943–952

A missense mutation in *rpoD* results in an A-signalling defect in *Myxococcus xanthus*

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Summary

The *Myxococcus xanthus asg* genes (*asgA*, *asgB*, and *asgC*) are necessary for production of extracellular A-signal, which is thought to function as a cell-density signal. Previous analyses of the *asgA* and *asgB* genes suggest that they perform regulatory functions. In this work, we localized *asgC* to a region that contains genes homologous to *rpsU*, *dnaG*, and *rpoD* of the *Escherichia coli* macromolecular synthesis (MMS) operon. Surprisingly, *asgC767* was found to be a mutant allele of *rpoD*, the gene encoding the major sigma factor of *M. xanthus*. The mutation in *asgC767* results in a glutamate to lysine substitution at amino acid 598, which lies within conserved region 3.1 of the major sigma factors. Previous studies have shown that the *asg* mutants share a number of growth and developmental phenotypes. We found that A-signal restores developmental expression of an A-signal-dependent gene (Ω4521) in the *asgC767* (*rpoDEK598*) mutant background in a manner similar to that seen in the *asgA* and *asgB* mutants. Because the *asg* mutants have very similar phenotypes and the *asg* genes encode proteins that appear to have regulatory functions, we hypothesize that the *asg* gene products function together in a regulatory pathway that is required for extracellular A-signal production.

Introduction

Myxococcus xanthus is a Gram-negative, rod-shaped bacterium that inhabits the soil. Under conditions of nutrient limitation and high cell density, tens of thousands of cells aggregate to form a mound-shaped fruiting body. Within this structure, cells differentiate to form ovoid myxospores that are metabolically quiescent and resistant to various environmental stresses (Kaiser and Losick, 1993; Shimkets, 1990).

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Cell–cell communication is required to co-ordinate the process of fruiting-body formation and myxospore differentiation. Five classes of mutants (*asg*, *bsg*, *csg*, *dsg*, and *esg*) that are unable to produce particular extracellular signals required for development have been identified (Downard *et al.*, 1993; Hagen *et al.*, 1978; Kroos and Kaiser, 1987). Each of these mutants is unable to release an extracellular signal, but is able to respond to the signal and develop when mixed with wild-type cells. The different classes of mutants were defined by the ability of one class of mutants to extracellularly complement the signalling defect of another class. (Downard *et al.*, 1993; Hagen *et al.*, 1978). Mutants within the same class are unable to develop when mixed, presumably because they are unable to produce the same signal. Because the correct timing and level of expression of developmentally regulated genes requires the sequential production of the extracellular signals, each mutant class is blocked at a different stage of development (Kaiser and Losick, 1993; Kroos and Kaiser, 1987).

The *asg* (A-signalling) mutants are blocked very early in development, before the cells have started to form aggregates. These mutants fail to release A-signal, which is a mixture of amino acids produced through extracellular proteolysis (Kuspa *et al.*, 1992a; Plamann *et al.*, 1992). The A-signal is thought to function as a cell-density signal during early development to ensure that there is a sufficient number of cells present to complete fruiting-body morphogenesis (Kuspa *et al.*, 1992b).

We would like to understand how the *asg* genes (*asgA*, *asgB* and *asgC*) are involved in A-signal production. The DNA sequences of both *asgA* and *asgB* have been reported and have provided information regarding their possible functions in production of the A-signal (Plamann *et al.*, 1994; 1995). The deduced amino acid sequence of AsgA is similar to the amino acid sequences of members of the histidine protein kinase (HPK) and the response regulator (RR) families of signal transduction proteins (for reviews see Parkinson, 1993; Parkinson and Kofoid, 1992; Stock *et al.*, 1989). Although there are many variations in these signal-transduction systems, the model system consists of an HPK that functions as a transmembrane sensor protein and an RR protein that mediates the cellular response to environmental signals. AsgA is unique in its domain organization, with a RR domain at the N-terminus and an HPK domain at the C-terminus. In addition, AsgA

appears to lack the hydrophobic transmembrane domain that is characteristic of many of the HPKs. We have proposed that AsgA functions within a phosphorelay that senses starvation and responds by altering expression of genes required for development, including those for the production of extracellular A-signal (Plamann *et al.*, 1995).

The deduced amino acid sequence of AsgB contains a putative DNA-binding helix-turn-helix (HTH). This HTH is located at the C-terminus of AsgB and is most similar to the HTH found within region 4 of the major sigma factors (Plamann *et al.*, 1994). Region 4 is thought to interact directly with the −35 region of promoters (Dombroski *et al.*, 1992; Gardella *et al.*, 1989; Siegele *et al.*, 1989). Other highly conserved regions typically found in sigma factors are absent in AsgB; therefore, it is unlikely that AsgB acts as a sigma factor. AsgB probably acts as a transcriptional activator and/or repressor that alters gene expression to control production of the A-signal.

In this work, we localized *asgC* to a region that contains genes homologous to *rpsU*, *dnaG*, and *rpoD* of the *Escherichia coli* macromolecular synthesis (MMS) operon (Burton *et al.*, 1983; Versalovic *et al.*, 1993). In addition, we found that the *asgC767* mutation lies within *rpoD*, which encodes the major sigma factor of RNA polymerase holoenzyme. This mutation results in a glutamate to lysine substitution within conserved region 3.1 of RpoD.

Results

Cloning of asgC *locus*

M. xanthus strain DK5115 contains a tetracycline-resistant version of the Tn*5* insertion Ω4561 (Kuspa and Kaiser, 1989), which is 66% co-transducible with *asgC*. DNA flanking this transposon was isolated using a method described previously (Gill *et al.*, 1988; Stephens and Kaiser, 1987). The resulting plasmids, pMSL100 and pMSL101, contain *M. xanthus* DNA flanking one side or the other of the transposon. pMSL100 and pMSL101, which contain approx. 17 kb and 26 kb of *M. xanthus* DNA, respectively, were used to transform the *asgC* mutant, DK767, to determine whether one of the plasmids contains the wild-type *asgC* allele. In order to restore the Asg^+ phenotype following homologous integration of the plasmid, the plasmid must contain the wild-type sequence at the site of the mutation and at least one intact end of the *asgC* gene. Restoration of the wild-type phenotype was tested by scoring the transformants for development on starvation (TPM) agar (see the *Experimental procedures*). Integration of plasmid pMSL100 restored development in all transformants tested, while integration of pMSL101 restored development in none of the transformants tested.

Subcloning of asgC

Further analysis of pMSL100 involved the identification of restriction fragments that, when cloned into pBGS18 and used to transform DK767, would rescue development (Fig. 1). Plasmid pLP48 contains a 9.7 kb *Hin*dIII fragment cloned into the vector pBGS18. It includes part of the IS*50* and the DNA immediately flanking the transposon insertion, and was found to rescue development of DK767. Plasmid pLPJ45, a *Hin*dIII–*Pst*I subclone of pLP48, was unable to rescue development of DK767. Exonuclease III digestion of pLP48 produced a set of nested deletions that were used to make a finer approximation of the site of the *asgC767* mutation. All of the deletion plasmids rescued development, although, as the insert became smaller, the percentage of Asg^+ transformants decreased. These results indicate that all plasmids except for pLPJ45 contain both the wild-type sequence at the site of the mutation and at least one intact end of the gene. We deduced that the *asgC767* mutation lies in the DNA that is in common to both pLP48.2F and pLP48.*Xba*I.

The nucleotide sequence of both strands of 7980 bp of DNA within the insert of plasmid pLP48 was determined. This sequence extends from upstream of the *Sma*I site through the distal *Hin*dIII site (Fig. 1A). A number of open reading frames (ORFs) were detected (Table 1 and Fig. 1A). Three of the ORFs are homologous to ORFs of the macromolecular synthesis (MMS) operon found in many bacterial species (Lupski and Godson, 1984; Versalovic *et al.*, 1993). The MMS operon contains genes that are necessary for the initiation of translation (*rpsU*), DNA replication (*dnaG*), and RNA synthesis (*rpoD*). The first gene of the operon, *rpsU*, encodes the ribosomal protein S21 which interacts with 16S ribosomal RNA sequences complementary to the ribosome-binding sites of mRNAs. Comparisons of the deduced amino acid sequences of the RpsU proteins of *M. xanthus*, *E. coli*, and *Bacillus stearothermophilus* are shown in Fig. 2A. *M. xanthus* RpsU is 71.9% similar and 53.1% identical to *E. coli* RpsU.

Initiation of DNA replication requires the activity of primase, the product of *dnaG*. This protein is responsible for the synthesis of RNA primers that are required for the production of lagging-DNA strand Okazaki fragments (Lupski and Godson, 1984; Versalovic *et al.*, 1993). A comparison of the deduced amino acid sequences of DnaG of *M. xanthus*, *E. coli*, and *Bacillus subtilis* is shown in Fig. 2B. *M. xanthus* DnaG is 56.4% similar and 35.3% identical to *E. coli* primase.

The *rpoD* gene encodes the major sigma subunit of RNA polymerase holoenzyme. Sigma factor is responsible for promoter sequence recognition during the initiation of mRNA synthesis. The *M. xanthus rpoD* gene was identified previously, and an amino acid sequence comparison between the major sigma factors from *M. xanthus*, *E.*

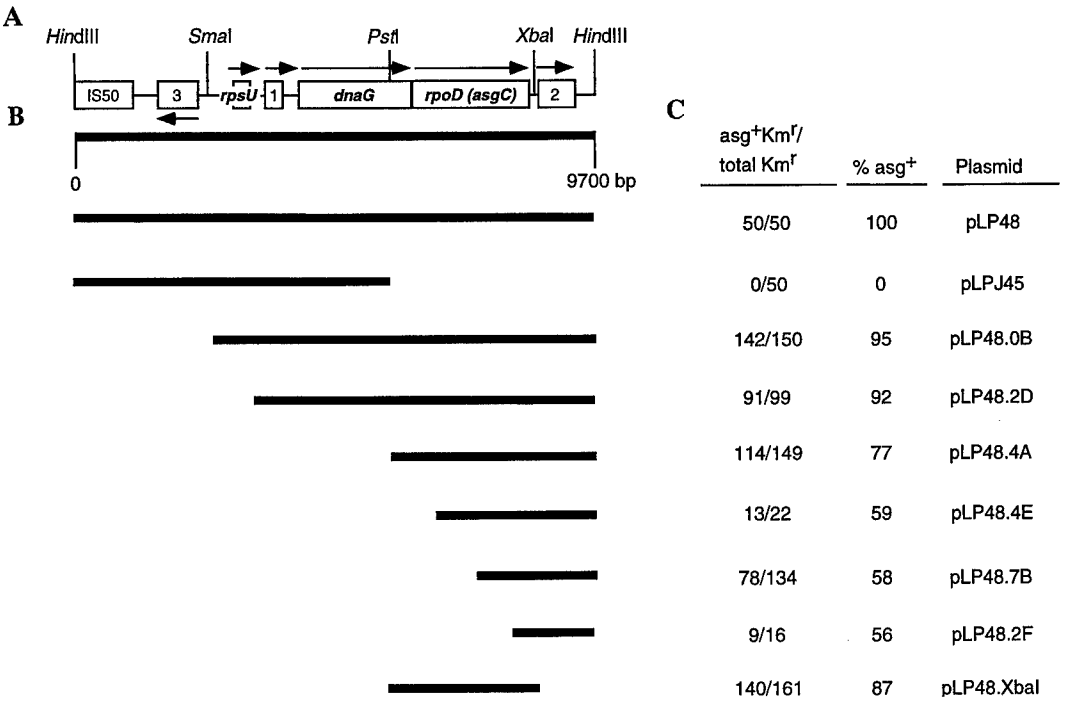

Fig. 1. Subcloning of *asgC*.
A. A 9.7 kb *Hin*dIII fragment from pMSL100 was found to phenotypically rescue the *asgC767* mutation when introduced into DK767 as part of an integrative plasmid (pLP48). The arrows above the map represent the ORFs of the *rpsU*, *dnaG* and *rpoD* genes, as well as two additional ORFs (ORF1 and ORF2, see Table 1 and the *Results*). A third additional ORF (ORF3) is in the opposite orientation and is located to the left of *rpsU* (see Table 1).
B. Exonuclease III digestions from the *Sma*I site produced the clones pLP48.OB, pLP48.2D, pLP48.4A, pLP48.4E, pLP48.7B, and pLP48.2F. Plasmid pLP48.*Xba*I was produced by *Xba*I digestion of pLP48.4A, removing the DNA sequences downstream of the *rpoD* ORF.
C. The percentages of *asg*$^+$ colonies produced from the integration of these plasmids into strain DK767 are to the left of the plasmid name as well as the number of *asg*$^+$ colonies over the number of colonies examined from each transformation.

coli, and *B. subtilis* has been published (Inouye, 1990). *M. xanthus* RpoD is 68.3% similar and 49.4% identical to RpoD of *E. coli.*

ORFs in addition to those of *rpsU*, *dnaG*, and *rpoD* were detected (Fig. 1 and Table 1). ORF1 is located between *rpsU* and *dnaG* and is not significantly similar to any ORFs in the database. ORF2 is located downstream of *rpoD*. The deduced amino acid sequence of ORF2 is very similar to Ogt (O^6-alkylguanine-DNA-alkyltransferase) of *E. coli* and Dat1 (O^6-methyl-guanine-DNA-alkyltransferase) of *B. subtilis* (Morohoshi *et al.*, 1989; Potter *et al.*, 1987). Ogt and Dat1 actively remove an O^6-methyl group from

Table 1. DNA sequence analysis.

ORF name	Start/stop codons[a]	No. of amino acids	% G+C in the third position[b]	Protein product[c]	Predicted size (kDa)	% similarity/ identity[d]
rpsU	1887(ATG)/2079(TAG)	64	82	Ribosomal protein S21	7.3	71.9/53.1
ORF1	2117(ATG)/2642(TGA)	175	87	?	18.5	?
dnaG	2976(GTG)/4795(TGA)	606	86	Primase	66.8	56.4/35.3
rpoD	4890(ATG)/7014(TGA)	708	94	Major sigma factor	80.4	68.3/49.4
ORF2	7040(GTG)/7481(TGA)	147	83	O6-alkylguanine-DNA-alkyltransferase ?	16	50.4/35.0
ORF3	1754(GTG)/164(TAG)[e]	530	89	Relaxase ?	56.4	40.8/20.9

The DNA sequence of 7.98 kb of *M. xanthus* DNA from pLP48 was determined and analysed using the Genetics Computer Group (GCG) software and has been assigned the GenBank accession number U20669.
a. The number of the first nucleotide of the start codon followed by the number of the first nucleotide of the stop codon. Numbering corresponds to the GenBank file.
b. Organisms with a high G+C content have, on average, a G or C in the third position of 89% of the codons (Bibb *et al.*, 1984; Shimkets, 1990).
c. A BLAST search (Altschul *et al.*, 1990) was performed to determine similarities of the deduced amino acid sequences of the ORFs to proteins in the database. Functions of these proteins are discussed in the *Results*.
d. Similarities and identities are based upon GAP alignments between *M. xanthus* and *E. coli* protein sequences.
e. ORF3 is in the opposite orientation relative to *rpsU*.

A

```
          1                                                              64
Mx RpsU  MPGIRVKEGE SIESALKRFK KATEKAGILS EIRKREHYEK PSVKRKKKAL AAKKRAVKKA RKSF
Ec RpsU   PVIKVRENE PFDVALRRFK RSCEKAGVLA EVRRREFYEK PTTERKRAKA SAVKRHAKKL ARENARRTRL Y
Bst RpsU  SKTIVRKNE SIDDALRRFK RAVSKTGTLQ EVRKREFYEK PSVRRKKKSE AARKRK
          p-i-VrenE sid-ALrRFK ra-eKaG-L- EvRkREfYEK Psv-RKkk-- aA-KR--kk
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B

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          1                                                                                80
Mx DnaG  VGVVIPEHKI QEVLERVDLV GLISRHVDLK KAGREWKACC PFHQEKTPSF YVVPEKRFYF CHGCRASGDA VSFVQRYLGK
Ec DnaG  MAGRIPRVFI NDLLARTDIV DLIDARVKLK KQGKNFHACC PFHNEKTPSF TVNGEKQFYH CFGCGAHGNA IDFLMNYDKL
Bs DnaG  MGNRIPDEIV DQVQKSADIV EVIGDYVQLK KQGRNYFGLC PFHGESTPSF SVSPDKQIFH CFGCGAGGNV FSFLRQMEGY
         mg-rIP---i --vl-r-DiV -lI---V-LK KqGrn--acC PFH-EkTPSF -V-peKqfyh CfGCgA-Gna -sFl--y-g-

          81                                                                               154
Mx DnaG  TFLDAVRDLA RELGVDLEAE ......QDPS MRERQQIKEA TDQAAEHFRA MLWQQDEGRS ARAYIASRGV SDETAMAFGL
Ec DnaG  EFVETVEELA AMHNLEVPFE .AGSGPSQIE RHQRQTLYQL MDGLNTFYQQ SLQQPVAT.S ARQYLEKRGL SHEVIARFAI
Bs DnaG  SFAESVSHLA DKYQIDFPDD ITVHSGARPE SSGEQKMAEA HELLKKFYHH LLINTKEGQE ALDYLLSRGF TKELINEFQI
         -F-e-V--LA -----d-p-e --------pe ---rQ---ea -d-l--fy-- -L-q--eg-s Ar-Yl-sRG- s-E-i--F-i

          155                                                                              233
Mx DnaG  GWAPLEWASL TERFQKLGML E.WAAKAGLV LKRNSGDGYY DFFRSRVMVP IRAPEGRPIA FGGRLIGADE GPKYLNSRES
Ec DnaG  GFAPPGWDNV LKRFGGNPEN RQSLIDAGML VTNDQGRSY. DRFRERVMFP IRDKRGRVIG FGGRVLGNDT .PKYLNSPET
Bs DnaG  GYALDSWDFI TKFLVKRGFS EAQMEKAGLL IRREDGSGYF DRFRNRVMFP IHDHHGAVVA FSGRALGSQQ .PKYMNSPET
         G-Ap--Wd-- tkrf-k-g-- e----kAGll --r--G-gY- DrFR-RVMfP Ird--Grvia FgGR-lG-d- -PKYlNSpEt

          234                                                                              313
Mx DnaG  RLYNKSETLF GMDQSRDEIR KRKAAVLVEG YFDALGLHQV GVRHAVALCS TNLTAGHMQV LKRAEARELI LLLDGDSAGL
Ec DnaG  DIFHKGRQLY GLYEAQQDNA EPNRLLVVEG YMDVVALAQY GINYAVASLG TSTTADHIQL LFRA.TNNVI CCYDGDRAGR
Bs DnaG  PLFHKSKLLY NFYKARLHIR KQERAVLFEG FADVYTAVSS DVKESIATMG TSLTDDHVKI LRRN.VEEII LCYDSDKAGY
         -lfhKs--Ly g-y-ar--ir kp-ravlvEG y-Dv--l-q- gv--avA--g TslTadH-q- L-Ra---e-A lcyDgD-AG-

          314                                                                              389
Mx DnaG  AAVERLSG.. .PLLAAGATA RVALLPQGDD PDTFARREGQ EGVE.RLLEG AHPLTSHLFA SLLPEGKAAS FEEKMAALER
Ec DnaG  DAAWRALETA LPYMTDGRQL RFMFLPDGED PDTLVRKEGK EAFEARMEQA .MPLSAFLFN SLMPQVDLST PDGRARLSTL
Bs DnaG  EATLKASEL. ..LQKKGCKV RVAMIPDGLD PDDYIKKFGG EKFKNDIIDA SVTVMAFKMQ YFRKGKNLSD EGDRLAYIKD
         -A--rase-- -pl---G--- Rva-lPdG-D PDt--rkeG- E-fe-r---a --pl-aflf- sl-p---ls- ---r-a----

          390                                                                              469
Mx DnaG  LKPVVGQVPV GLVRATLFSA VAEHFGWRPA DVEAALRSKV PLPKPAGGDA PPSSPNRPAP PLEKPPPALE CFYVGAVLKE
Ec DnaG  ALPLISQVPG ETLRIYLRQE LGNKLGILDD SQLERL.... .MPKAAE... ..SGVSRPVP QLKRTTM... ..........
Bs DnaG  VLKEISTLSG SLEQEVYVKQ LASEFSLSQE SLTEQLSVFS KQNKPAD... ..NSGETKTR RAHLTTKARQ KRLRPAYENA
         -lp-isqvpg -l-r--l--- la--fg---- s--e-L---- --pKpA---- --ss--rp-p -l--tt-a-- -----a----

          470                                                                              547
Mx DnaG  PRLMARDTFR VCDELSHMGL RMALAHATSG HGANDALFES SEAVKRGIES A..LRQLPSE PVPLEAAFLS ICREIMVRRI
Ec DnaG  .RILIGLLVQ NPELATLVPP LENLDENKLP GLGLFRELVN TCLSQPGLTT GQLLEHYRGT NNAATLEKLS MWDDIADKNI
Bs DnaG  ERLLLAHMLR DRSVIKKVID RVGFQFNIDE HRALAAYLYA FYEEGAELTP QHLMARVTDD HISQLLSDIL MLQ..VNQEL
         -R1l-----r -------v-- r--l--n--- h-al-a-l-- ------glt- --ll------ -----l--ls m---i----i

          548                                                          606
Mx DnaG  DERLV..YIK RATEQTPGAF DLTEETRQLL VERVELLALK KRVLEELKPA SSGTKAPMQP V
Ec DnaG  AEQTFTDSLN HMFD....SL LELRQEELIA RERTHGLSNE ERL..ELWTL NQELAKK
Bs DnaG  SEAELSDYVK KVLNQRNWSM IKEKEAERAE AERQKDFLRA ASLAQEIVTL NRSLK
         -E----dy-k ----q---s- ----e-e--- -ER---l--- -rl--El-tl n--lk
```

C

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                                                                 1                    24
Mx ORF                                                           VGPRSI RGPGAFV..F LESGAVALRA
Ec OGT   MLRLLEEKIA TPLGPLWVIC DEQ....FRL RAVEWEEYSE AMVQLLDIHY RKEGYERISA TNPGGLSDKL RDYFAGNLSI
Bs DAT   ..MNYYTTAE TPLGELIIAE EEDRITRLFL SQEDWVDWKE TV........ ..QNTEHKET PNLAEAKQQL QEYFAGERKT
                   tplg-l---- -e-------l ----w----e ---------- -----e--s- -npg-----l -eyfAg-l--

         25                                                                              102
Mx ORF   KTKTPKAEVK KAPLPFSKAV WKAVRAIPRG QVRSYAQVAL YAGRPGAARG VGREMATLPQ QPELPLPWWR VTRSDGTL..
Ec OGT   IDTLPTAT.. .GGTPFQREV WKTLRTIPCG QVMHYGQLAE QLGRPGAARA VGA..ANGSN PISIVVPCHR VIGRNGTMTG
Bs DAT   F.SLPLSQ.. .KGTPFQQKV WQALERIPYG ESRSYADIAA AVGSPKAVRA VGQ..ANKRN DLPIFVPCHR VIGKNSALTG
         ---lP-a--- --gtPFq--V Wkalr-IP-G qvrsYaq-A- --GrPgAaRa VG---An--n ---i-vPchR Vig-ngtltg

         103                                                147
Mx ORF   .APQVAQEQA RRLRAEGVEV TQRGDTFRVK VPREVPAPGL ATKRQR
Ec OGT   YAGG.VQRKE WLLRHEGYLL L
Bs DAT   YAGSKTEIKA FLLNIERISY KEK
         yAg---q-ka -lLr-Eg
```

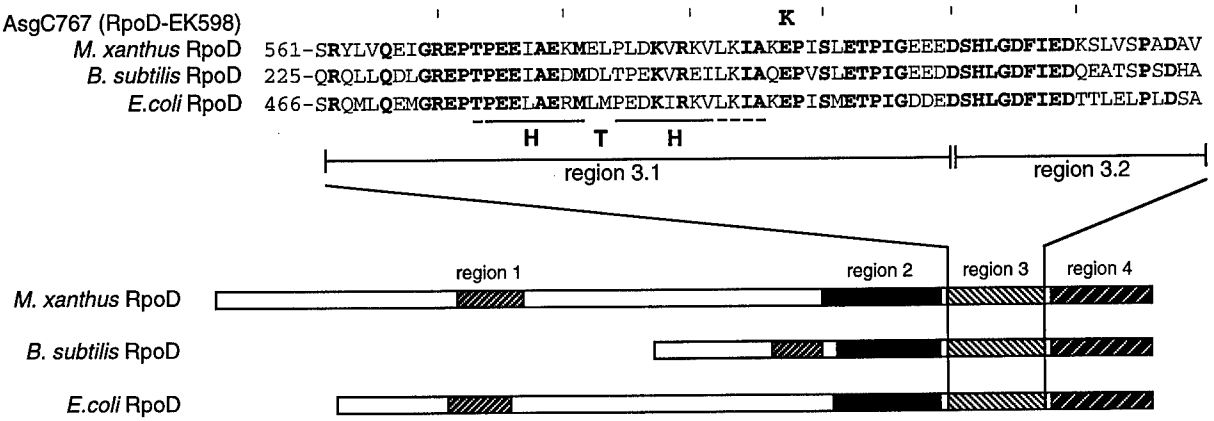

Fig. 3. Analysis of the *asgC767* allele. The amino acid sequences of region 3 of RpoD of *M. xanthus*, *B. subtilis* and *E. coli* are presented above a diagrammatic representation of the conserved regions within the major sigma factors. Subregions 3.1 and 3.2 are indicated below the amino acid sequences. The result of the *M. xanthus asgC767* missense mutation is a glutamate to lysine substitution at position 598 within subregion 3.1. The amino acid change in AsgC767 is indicated above the amino acid sequences. The region that resembles a HTH is indicated below the amino acid sequences (Lonetto *et al.*, 1992).

O^6-methylguanine and transfer this group to a cysteine residue within the enzyme (Morohoshi *et al.*, 1989; Potter *et al.*, 1987). The deduced *M. xanthus* ORF is 50.4% similar and 35.0% identical to Ogt of *E. coli*. A comparison between the deduced amino acid sequences of ORF2, Ogt of *E. coli* and Dat1 of *B. subtilis* is shown in Fig. 2C.

ORF3 is located upstream of *rpsU*, and is in the opposite orientation relative to *rpsU*. The deduced amino acid sequence of ORF3 is 40.8% similar and 20.9% identical to the conjugation protein TraI of *E. coli* (Ziegelin *et al.*, 1991). *traI* encodes a relaxase necessary for relaxasome formation and site- and strand-specific cleavage at the transfer origin (oriT) (Balzer *et al.*, 1994).

Cloning and sequence of the asgC767 *allele*

The *asgC767* allele was isolated using the method described by Mayo and Kaiser (1989) for the isolation of *asgB480*. In this method, plasmid pMSL100 (*asgC*$^+$) was introduced into DK767 (*asgC767*) by electroporation, and kanamycin resistant (Km^R) colonies were selected. After pMSL100 integrates into the chromosome by homologous recombination at the *asgC* locus, gene conversion may occur, resulting in two possible phenotypes: Asg^+/Km^R (developmentally competent) or Asg^-/Km^R (developmentally incompetent).

Chromosomal DNA was purified from two Asg^-/Km^R colonies, digested with *Eco*RI, ligated and used to transform *E. coli*. Plasmids pLPJ58 and pLPJ59 were isolated from Km^R *E. coli* colonies. Southern hybridization (data not shown) with plasmids pLPJ58 and pLPJ59 confirmed that they contain DNA homologous to the *asgC*$^+$ insert of parent plasmid pMSL100. As expected, when they were used to transform DK767, no Asg^+ colonies resulted. The 9.7 kb *Hin*dIII fragments from pLPJ58 and pLPJ59 were cloned into pBGS18 to produce plasmids pLPJ60 and pLPJ61. The DNA sequence was determined for the region within *rpoD* that we deduced from the data in Fig. 1B to contain the *asgC767* mutation. We found that the *asgC767* allele contains two transition mutations (G to A) at nucleotides 6680 and 6681 within *rpoD* . Codon 597 incurs a silent change of AAG to AAA (both encoding lysine) and codon 598 has a missense mutation (GAG to AAG) resulting in a glutamine to lysine substitution (Fig. 3). This mutation, which we will now call *rpoDEK598*, lies within the coding sequence for region 3.1 of the major sigma factor. This region contains many acidic residues and is conserved among σ^{70}-like sigma factors (Lonetto *et al.*, 1992).

The asgC767 *mutant responds to proline and trypsin in A-factor assays*

Previous studies have shown that the *asg* mutants share a

Fig. 2. Comparisons between the deduced amino acid sequences of *M. xanthus* RpsU, DnaG, and ORF2 and their homologues in *E. coli* and *B. stearothermophillus* or *B. subtilis*.
A. Comparison of RpsU sequences of *M. xanthus* (Mx), *E. coli* (Ec) (Burton *et al.*, 1983), and *B. stearothermophillus* (Bst) (Herfurth *et al.*, 1991).
B. Comparison of DnaG sequences of *M. xanthus*, *E. coli* (Burton *et al.*, 1983), and *B. subtilis* (Bs) (Wang and Doi, 1986).
C. Comparison of the ORF2 sequence of *M. xanthus* with the Ogt sequence of *E. coli* (Potter *et al.*, 1987), and the Dat1 sequence *B. subtilis* (Morohoshi *et al.*, 1989). Lower-case letters under the sequence alignments are residues that are identical in two of the three amino acid sequences, upper-case letters are residues that are identical in all three amino acid sequences.

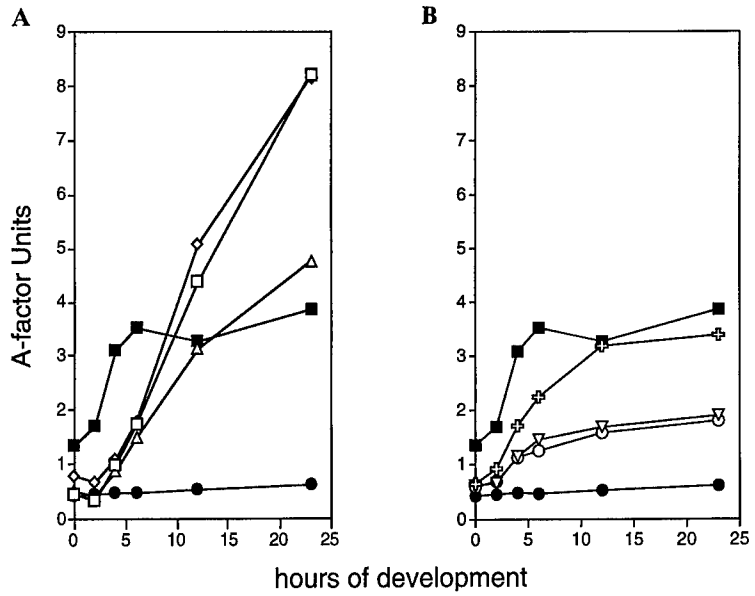

Fig. 4. Effect of proline and trypsin on expression of Ω4521 in the *asgC* mutant LP110. Expression of the A-signal-dependent Tn*5lac* insertion Ω4521 was measured in A-factor units in wild-type *M. xanthus* (■), and LP110 with either: A. no addition (●), 0.5 mM proline (△), 1.0 mM proline (◇), and 2.0 mM proline (□), or B. no addition (●), 0.5 μg ml^{-1} trypsin (○), 1.0 μg ml^{-1} trypsin (▽), 5.0 μg ml^{-1} trypsin (✠). One unit of A-factor activity is defined as the amount required to stimulate the test cells to produce 1 U of β-galactosidase activity (1 nmol min^{-1} of *o*-nitrophenol) above background (Plamann *et al.*, 1992). Data points are the means of three experiments performed in triplicate, with the exception of the time point at 12 h, which is the mean of two experiments.

number of growth and developmental phenotypes. One of the developmental phenotypes exhibited by the *asgA* and *asgB* mutants is decreased expression of an A-signal-dependent Tn*5lac* insertion, Ω4521 (Kuspa *et al.*, 1986; Plamann *et al.*, 1995). We found that, as in *asgA* and *asgB* mutants, expression of Ω4521 is reduced during growth and development in the *asgC767* background (Fig. 4).

The A-factor (A-signal) assay was used to identify substances that have the ability to restore developmental expression of Ω4521 in an *asgB* mutant. Proline and trypsin, as well as other amino acids and proteases, have activity in A-factor assays (Kuspa *et al.*, 1992a, b; Plamann *et al.*, 1992). We tested the ability of proline and trypsin to restore developmental expression of Ω4521 in the *asgC767* background (using strain LP110). Proline, at a concentration of 200 μM, increases expression of Ω4521 in an *asgB480* background approx. fivefold (Kuspa *et al.*, 1992a). We found that 500 μm proline increases expression of Ω4521 in the *asgC767* background approx. fivefold (compared to the level observed in the wild-type background) after 12 h of development (Fig. 4A). Higher concentrations of proline (1 mM and 2 mM) significantly increased expression of Ω4521.

Trypsin has the highest A-factor activity of all proteases tested. Approximately 0.5–1.0 μg ml^{-1} trypsin restores expression of Ω4521 to the wild-type level in an *asgB* mutant (Plamann *et al.*, 1992). Trypsin at concentrations of 0.5, 1.0, and 5.0 μg ml^{-1} was tested for its ability to rescue expression of Ω4521 in strain LP110 (Fig. 4B). Trypsin at 5 μg ml^{-1} increased expression of Ω4521 to the wild-type level after 12 h of development. Lower concentrations of trypsin did not completely rescue expression. These results indicate that the *asgC767* (*rpoDEK598*) mutant is similar to the *asgA* and *asgB* mutants with respect to Ω4521 expression and rescue by the A-signal.

Discussion

The DNA sequence analysis shows that the *M. xanthus rpoD* gene lies downstream of genes homologous to *rpsU* and *dnaG* of *E. coli*. *rpsU*, *dnaG*, and *rpoD* encode proteins necessary for the initiation of protein, DNA, and mRNA synthesis, respectively (Versalovic *et al.*, 1993). These genes make up the MMS operon in *E. coli*, and are contiguous within a number of bacterial species. It is likely that the *rpsU*, *dnaG*, and *rpoD* genes of *M. xanthus* are also arranged in an operon, although this remains to be determined. We detected three additional ORFs in this region. One ORF of unknown function is located between *rpsU* and *dnaG*. A second ORF begins 26 bp downstream of *rpoD* and is predicted to encode a protein that is 35% identical to *E. coli* Ogt (Potter *et al.*, 1987). Because the intergenic region between ORF2 and *rpoD* is short, it is possible that ORF2 is a member of this putative operon. If ORF1 and ORF2 are part of the putative *M. xanthus* MMS operon, the gene arrangement differs from other Gram-negative bacteria that have been examined. In particular, in nine out of 13 different Gram-negative species examined, *rpsU* and *dnaG* are contiguous. In the remaining four species, the genes are separated by ERIC (Enterobacterial Repetitive Intergenic Consensus) DNA sequences (Versalovic *et al.*, 1993). In contrast, the *M. xanthus rpsU* and *dnaG* genes are separated by ORF1, which has the potential to encode a 175-amino-acid polypeptide (Table 1). The third ORF is located upstream of,

and in the opposite orientation relative to, *rpsU*. Its putative protein product is 20.9% identical to the conjugation protein TraI of *E. coli* (Ziegelin *et al.*, 1991).

The *asgA*, *asgB*, and *asgC* (*rpoD*) genes are required for extracellular A-signal production (Kuspa and Kaiser, 1989). The A-signal, which is a mixture of amino acids and peptides (Kuspa *et al.*, 1992a; Plamann *et al.*, 1992), is thought to act as a cell-density signal that allows *M. xanthus* to determine whether there is a sufficiently high cell number for successful fruiting-body formation (Kuspa *et al.*, 1992b). The *asg* mutants are all defective in aggregation and sporulation, presumably because of a reduction in A-signal release. The defects are most severe for *asgA*, intermediate for *asgB*, and least severe for *asgC* (Kuspa and Kaiser, 1989). Addition of A-signal to the *asgA* and *asgB* mutants restores expression of an A-signal-dependent gene, Ω4521 (Kuspa *et al.*, 1992a; Plamann *et al.*, 1992). In this work, we found that A-signal (proline or trypsin) restores developmental expression of Ω4521 in the *asgC* (*rpoD*) mutant in a manner similar to that seen in an *asgB* mutant (Kuspa *et al.*, 1992a).

Analysis of *asgA* and *asgB* suggests that these genes encode regulatory proteins. AsgA is a kinase (Plamann *et al.*, 1995; Li and Plamann, 1996) that contains domains conserved among the HPK and RR proteins of two-component regulatory systems. *asgB* encodes a putative DNA-binding protein (Plamann *et al.*, 1994). We show here that the *asgC767* mutation lies within *rpoD*, the gene encoding the major sigma factor of *M. xanthus*. Sigma is the subunit of RNA polymerase that is required for specific promoter recognition. Interestingly, the *asgC767* (*rpoDEK598*) mutant does not appear to have a general defect in growth, and its vegetative phenotypes (tan rather than yellow colony colour, decreased extracellular enzyme production, and decreased cohesiveness) are very similar to those of the *asgA* and *asgB* mutants (Kuspa and Kaiser, 1989). Given that the *asg* mutants have very similar phenotypes and the *asg* genes encode proteins that appear to have regulatory functions, we hypothesize that the *asg* gene products function together in a regulatory pathway that is required for production of extracellular A-signal. Alternatively, the *asg* gene products may function in different, but related, regulatory pathways.

The *asgC767* (*rpoDEK598*) mutation consists of two consecutive base substitutions that result in a single amino acid change (glutamate to lysine) at amino acid 598. This change is located within conserved region 3, which is one of four highly conserved regions shared by sigma factors (Lonetto *et al.*, 1992). The two most highly conserved are regions 2 and 4. The proposed functions of region 2 include recognition of DNA sequences at the −10 region of the promoter, interactions with core RNA polymerase, and DNA-strand opening (Helmann and Chamberlin, 1988; Lonetto *et al.*, 1992). The proposed function of region 4 involves recognition of the −35 sequences of the promoter (Gardella *et al.*, 1989; Siegele *et al.*, 1989). There is evidence that region 1 is involved in inhibition of DNA binding by a free sigma subunit (Dombroski *et al.*, 1992). Region 3 is divided into 2 subregions. Subregion 3.2 may be involved in core RNA polymerase binding. Subregion 3.1 contains a sequence that weakly resembles a HTH domain (Lonetto *et al.*, 1992). The mutation in *asgC767* (*rpoDEK598*) occurs just downstream of this putative HTH.

Mutations that affect interactions between RNA polymerase and transcription factors have been identified in the genes encoding the alpha and sigma subunits of RNA polymerase (Ishihama, 1993). *E. coli rpoD* mutations that affect interactions with the transcription factors PhoB (Makino *et al.*, 1993), cAMP receptor protein (at the P1*gal* promoter) (Kolb *et al.*, 1993), AraC (Hu and Gross, 1985), and the λ *c*I repressor (Kuldell and Hochschild, 1994; Li *et al.*, 1994) have been localized to region 4. Kumar *et al.* (1994) suggest, from data provided through deletional analysis of the C-terminal portions of *rpoD*, that a region extending from at least region 3.2 to upstream of region 4.2 may be involved in association with transcription factors. Recently, Bramucci *et al.* (1995) identified a mutation in a *B. subtilis* sigma-factor gene (*spo0H*) that suppresses the transcriptional defects of a mutant form of the transcription factor Spo0A (Spo0A9V). This *spo0H* mutation is located between the sequences encoding regions 2 and 3 and is proposed to allow Spo0A9V to interact with the mutant σ^H, restoring transcriptional activation. Similarly, the A-signalling defect observed in the *rpoDEK598* strain may be caused by a failure of the mutant sigma subunit to productively interact with a transcriptional regulator necessary for A-signal production. If this hypothesis is correct, the isolation and characterization of suppressors of *rpoDEK598* may allow identification of the hypothetical transcriptional regulator or its target genes. An alternative hypothesis is that the mutant sigma factor has a greater affinity for core polymerase, preventing alternative sigma factors necessary for A-signal production from gaining access to the core. In this case, a suppressor analysis may result in the identification of genes encoding alternative sigma factors or RNA-polymerase mutants that have altered affinities for sigma subunit.

Experimental procedures

Bacterial strains, plasmids, and phage

M. xanthus strain DK101 carries the social-motility mutation *sglA1* and is used in all experiments as the wild-type strain (Hodgkin and Kaiser, 1977). DK767 carries both the *sglA1* and *asgC767* mutations (Kuspa and Kaiser, 1989). Strain DK4561 carries the *sglA1* mutation as well as a Tn*5* transposon insertion (Ω4561) that is linked to the *asgC767* locus

(Kuspa and Kaiser, 1989). DK5115 is a Tc^R version of DK4561. Strain LP110 contains the Tn*5lac* insertion Ω4521 in an *asgC767 sglA1* background. *E. coli* strain DH10B (BRL) was used for the growth and maintenance of plasmids. Plasmid pKNS116 is a pBR322-derived plasmid containing the Km^R gene and IS*50L* from Tn*5* (Mayo and Kaiser, 1989). pBGS18 is a Km^R analogue of pUC18 (Spratt *et al.*, 1986). Plasmids pMSL100 and pMSL101 contain DNA flanking the Tn*5* insertion Ω4561 in DK5115. Plasmid pLP48 contains the 9.7 kb *Hin*dIII fragment from pMSL100 in the pBGS18 vector. pLPJ45 contains a 5 kb *Hin*dIII–*Pst*I fragment from pLP48 in pBGS18 vector. Plasmids pLP48.0B, pLP48.2D, pLP48.4A, pLP48.4E, pLP48.7B, and pLP48.2F are plasmids derived from Exonuclease III digestion of pLP48. Prior to the Exonuclease III digestion, 3 pmol of twice-CsCl-purified pLP48 was digested first with *Kpn*I and then by *Sma*I. The Exonuclease III digestion was performed as described by Sambrook *et al.* (1989). The resulting plasmids were analysed by restriction-enzyme digestion to determine the size of the remaining insert DNA. pLP48.*Xba*I was created by removing an approximately 900 bp *Xba*I fragment just downstream of the *rpoD* ORF from plasmid pLP48.4a. Plasmids pLPJ58 and pLPJ59 are analogous to pMSL100 except that they carry the mutant allele *asgC767*. pLPJ60 and pLPJ61 contain the 9.7 kb *Hin*dIII fragments from pLPJ58 and pLPJ59, respectively, in pBGS18. P1*cam clr* - 100 (Rosner, 1972) was used as a specialized transducing phage to introduce DNA from *E. coli* to *M. xanthus* in the original cloning of the *asgC* locus. The myxophage Mx4 *hrm ts18ts27* (Geisselsoder *et al.*, 1978) is a generalized transducing phage of *M. xanthus* and was used to transduce the Tn*5lac* insertion Ω4521 from strain DK4322 (Kuspa and Kaiser, 1989) into DK767 to construct LP110.

Growth and development

M. xanthus strains were grown at 32°C in CTT broth (1% casitone (Difco), 10 mM Tris-HCl (pH 7.6), 1 mM KH_2PO_4 (pH 7.7), and 8 mM $MgSO_4$) or on CTT agar (CTT broth with 1.5% Bacto agar (Difco)) supplemented with kanamycin sulphate to a final concentration of 40 μg ml^{-1}, when appropriate. Development was carried out on TPM agar (CTT agar without the casitone).

DNA manipulations

Standard procedures for restriction-endonuclease digestions, agarose-gel electrophoresis, purification of DNA from low-melting-point agarose gels, DNA ligations, and other cloning-related techniques were performed as described by Sambrook *et al.* (1989). Southern hybridizations were performed using a radioisotope-free system (Schleicher and Schuell). Electroporation of plasmid DNA into *M. xanthus* was performed as described by Kashefi and Hartzell (1995).

Isolation of chromosomal DNA

Chromosomal DNA was purified using a modified form of the method of Laue and Gill (1994). Cells from 5 ml cultures of *M. xanthus* (approximately 2.5×10^9 cells) were resuspended in 3 ml of sucrose–Tris buffer (25% sucrose, 125 mM EDTA, 0.5 M Tris (pH 8)); 0.6 ml of lytic mixture (5% SDS, 125 mM EDTA, 0.5 M Tris (pH 9.6)) was then added and the samples were heated at 70°C for 60 min. Pronase (10 mg ml^{-1} in TE) was preheated at 37°C for 60 min then 0.4 ml TE was added to the cells. The mixture was incubated at 37°C overnight and then phenol:chloroform (10:1) extracted 3 times. Nucleic acids were precipitated by addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of room-temperature 100% ethanol. Pellets were resuspended in 1 ml of TE containing 10 μg ml^{-1} RNase and then incubated for 30 min at 37°C. One-tenth volume of 3 M sodium acetate was added to the DNA, and 0.625 volumes of ice-cold isopropanol was layered on top. DNA was harvested by spooling with a bent Pasteur pipette, washed with 70% ethanol and resuspended in 50 μl of H_2O.

DNA sequence and computer analysis

DNA sequence was determined by the dideoxy chain-termination method (Sanger *et al.*, 1977), using the SequiTherm Cycle Sequencing Kit (Epicentre Technologies) and custom oligonucleotide primers synthesized by the Gene Technologies Laboratory at Texas AandM University. The DNA sequence was assembled and analysed using software from the Genetics Computer Group (1991) and CodonUse 3.0 (1993). GenBank searches were performed at the National Center for Biotechnology Information, using the BLAST network services (Altschul *et al.*, 1990).

Cloning the asgC$^+$ *locus*

Cloning of the wild-type *asgC* locus followed protocols described previously (Gill *et al.*, 1988; Stephens and Kaiser, 1987). Plasmid pKNS116, a derivative of plasmid pREG429, was introduced into *M. xanthus* DK5115, an otherwise wild-type strain carrying the Tc^R version of Tn*5* insertion Ω4561, which was shown to be 66% cotransducable with *asgC767* (Kuspa and Kaiser, 1989). This plasmid integrated into the *M. xanthus* chromosome by homologous recombination with one of the two IS*50*s of the resident transposon. Chromosomal DNA was isolated as described above and digested with *Eco*RI endonuclease, ligated in 100 μl of ligase buffer (i.e. in dilute solution) to promote intramolecular ligation, and used to transform *E. coli*. Km^R colonies were then selected and, from these, plasmid DNA was isolated. The two resulting plasmids, one containing *M. xanthus* DNA flanking the arbitrarily designated right-hand side of the Ω4561, and the other containing DNA flanking the arbitrarily designated left-hand side of Ω4561, were named pMSL100 and pMSL101, respectively.

Cloning the asgC767 *allele*

The *asgC767* allele was cloned by the method described by Mayo and Kaiser (1989) for the cloning of *asgB480*. Plasmid pMSL100 (*asgC*$^+$) was introduced into *M. xanthus* by electroporation, and cells were plated onto CTT agar containing 40 μg ml^{-1} kanamycin. Partial diploids were formed by the integration of pMSL100 into the DK767 chromosome at the *asgC767* locus. Asg^- segregants were identified by testing

the transformants for the ability to develop on TPM agar. Chromosomal DNA was isolated from two Asg$^-$ segregants, then cut with *Eco*RI, ligated and used to transform *E. coli*. Resulting plasmid DNAs, pLPJ58 and pLPJ59, were used in a Southern hybridization experiments, along with a 1.5 kb *Bam*HI–*Xho*I fragment, from within the *rpoD* ORF, as probe to determine whether the plasmids contain the *rpoD* locus. Both pLPJ58 and pLPJ59 were used to transform DK767 to test whether they could rescue development. A 9.7 kb *Hin*dIII fragment corresponding to the *M. xanthus* insert DNA in pLP48 was subcloned from these plasmids into pBGS18 to produce pLPJ60 and pLPJ61.

A-factor assay

A-factor assays were performed as described by Plamann *et al.* (1992) with the exception that LP110 was used as the tester strain. One unit of A-factor activity is defined as the amount required to stimulate the test cells to produce 1 U β-galactosidase activity (1 nmol min^{-1} of *o*-nitrophenol) above background.

Nucleotide sequence accession number

The 7980 bp DNA sequence has been assigned GenBank accession number U20669.

Acknowledgements

We wish to thank Drs Margaret S. Lee and Dale Kaiser for their generous gift of plasmids pMSL100 and pMSL101. We also thank Drs Susan Golden, Mike Plamann, and Debby Siegele for helpful discussions concerning the manuscript. This investigation was supported, in part, by Public Health Service research Grant GM47265 from the National Institutes of Health.

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Appendix B

Cell-Density Sensing during Early Development in *Myxococcus xanthus*

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Introduction

Myxobacteria are motile, gram-negative, rod-shaped bacteria that inhabit diverse soil types the world over. These bacteria glide over solid surfaces, colonizing habitats such as decaying leaves, rotting wood or the dung of herbivores (Reichenbach, 1993). Although myxobacteria were originally classified as fungi, they are now widely recognized as unique, gram-negative bacteria that exhibit complex social behaviors. Observation of these social behaviors has inspired researchers to investigate the nature of the cell-cell interactions and signaling phenomena that coordinate them.

The selective force behind the social behaviors of *Myxococcus xanthus* is thought to be its unusual mode of cooperative feeding (Dworkin, 1973). Swarms of *M. xanthus* cells, sometimes referred to as "microbial wolf packs ," release antibiotics to kill neighboring competing species and break down their cell walls and other macromolecules using a battery of hydrolytic enzymes. The protein contents of these prey microorganisms provides the major source of carbon and energy for the myxobacteria. Feeding as part of a swarm allows the individual cell to grow more rapidly because the extracellular concentration of hydrolytic enzymes reaches a higher level within a swarm, resulting in a higher concentration of transportable nutrients (Dworkin, 1973, Kaiser, 1984).

Fruiting body formation of *M. xanthus* is the myxobacterial social behavior that has been the most extensively studied. When *M. xanthus* cells are starving and at a high cell density, approximately 100,000 cells glide to aggregation centers where they participate in the formation of multicellular, haystack-shaped fruiting bodies. Cells within nascent fruiting bodies differentiate from rod-shaped cells into ovoid, heat- and desiccation-resistant myxospores. Formation of fruiting bodies probably aids in the dispersal of myxospores and ensures that when spores germinate in the presence of a suitable food supply, there will be a sufficiently high

number of cells to participate in cooperative feeding (Dworkin and Kaiser, 1985, Kaiser, 1984).

It has long been known that fruiting body formation in *M. xanthus* requires nutrient limitation, a high cell density, and a solid surface. How do cells sense these conditions and then synchronize the initiation of development? How do cells coordinate their movements toward aggregation centers? How do cells achieve specific alignments to construct a fruiting body with the characteristic haystack-shape? Although progress has been made toward finding answers to these questions, much remains to be learned.

This chapter covers the advances that have been made in the area of cell density sensing during early development in *M. xanthus* It begins with an overview of work on cell-cell signaling that led to the identification of a cell-density signal. This signal, A signal, is composed of a specific set of extracellular amino acids that are generated through the action of extracellular proteases (Kuspa et al., 1992). The most recent research has focused on understanding the signal transduction mechanisms that regulate A-signal generation and mediate the response to A signal.

Isolation of cell-cell signaling mutants

The genetic experiments that opened the door to studies on cell-cell signaling in myxobacteria were carried out by Hagen *et al.* (Hagen et al., 1978) in Dale Kaiser's laboratory at Stanford University. These experiments were an extension of earlier work by McVittie, Messik, and Zahler (McVittie et al., 1962) , who observed that mixing pairs of particular developmental mutants results in normal sporulation. It was reasoned that if intercellular signals are used by myxobacteria to coordinate fruiting body formation and sporulation, then it should be possible to isolate mutants that fail to release molecular signals and, therefore, do not carry out

these processes. Such mutants should be able to fruit and sporulate when mixed with cells that are capable of producing the missing molecules. A simple genetic screen was devised to isolate the conditional, nonsporulating mutants (Hagen et al., 1978). Mutagenized cells were plated at a high cell density on agar plates that promote fruiting. After allowing fruiting bodies to form, any remaining vegetative (heat-sensitive) cells were killed by heating the plates. Myxospores were plated to form single colonies on clone-fruiting agar. Under these conditions, myxospores germinate and form a thin colony. Once the thin colonies have formed, the cells have exhausted the nutrient supply and fruiting bodies form within the colony. The conditional mutants-- those that sporulate only in the presence of signal-producing cells-- fail to form fruiting bodies on these plates.

After isolating approximately 50 non-sporulating, conditional mutants using this protocol, the question arose whether all of these mutants are defective in the same function. To test this, the conditional mutants were placed in pair-wise mixtures to assay for sporulation. The prediction is that synergism (sporulation) will occur only in mixtures comprised of two mutants that fail to make different signals. These experiments led to the identification of four groups of mutants (groups A, B, C, and D), with each group predicted to be defective in producing an intercellular signal required for normal fruiting and sporulation. Of the mutants that could be classified in this manner, approximately half belong to group A (Hagen et al., 1978). The remainder of this chapter is focused on the group A mutants, which we now know are defective in production of extracellular A signal.

A timeline of development

The group A mutants (from here on referred to as the *asg* mutants for A-signal-generating mutants) exhibit defects early in development. On solid clone-fruiting medium, the mutant cells form irregular, loose mounds that do not contain

myxospores (LaRossa et al., 1983). This same description could be used to explain the developmental phenotypes of the groups B, C, and D mutants; therefore, visual examination of developing cultures provides little information regarding the relative times of developmental arrest. As a first step in pinpointing more precisely the times of arrest within the developmental pathway, La Rossa *et al.* (LaRossa et al., 1983) examined the mutants for the presence of certain development-specific molecules: Guanosine tetra- and pentaphosphate [(p)ppGpp], protein S, and MBHA. There is considerable evidence that (p)ppGpp acts as a starvation signal in *M. xanthus* (Singer and Kaiser, 1995), and it is thought that accumulation of (p)ppGpp may be one of the earliest events in fruiting. All of the conditional mutants tested, including the *asg* mutants, accumulate (p)ppGpp following transfer of the cells from a rich medium to one lacking nutrients, indicating that the mutants were at least able to initiate a response to starvation. However, some aspect of starvation sensing or response appears to be defective in the *asg* mutants. When wild-type cells are placed on solid minimal medium lacking phenylalanine, growth ceases and fruiting bodies form. Under these same conditions, the *asg* mutants (with one exception) continue to grow, as if they have lost the ability to respond to nutrient limitation (LaRossa et al., 1983).

Protein S is a spore coat protein that normally is produced 6 h after the initiation of development; MBHA, a lectin, is normally produced approximately 12 to 15 h into development. La Rossa *et al.* (LaRossa et al., 1983) found that the *asg* mutants delay production of protein S and fail to produce MBHA. These studies placed the point of developmental arrest in the *asg* mutants sometime before 6 hr on the 24 hr timeline of development. The other groups of conditional mutants appeared to arrest at later points in the developmental pathway.

Although the above results provided a rough estimate of the times of developmental arrest and confirmed the finding that there are at least four classes

of conditional mutants, more developmental markers were needed to realize the full potential of these experiments. To this end, Kroos and Kaiser (Kroos and Kaiser, 1984) constructed a transposable promoter probe, Tn5 *lac*, which generates transcriptional fusions to *lacZ* when it transposes in the proper orientation into a gene. This advance, along with gene delivery methods utilizing P1 phage (Kuner and Kaiser, 1981, O'Connor and Zusman, 1983, Shimkets et al., 1983), brought the field of myxobacterial development into the age of molecular genetics. The promoter probe was used to identify a number of genes that are induced at various times during development and whose expression increases at least 3-fold following induction (Kroos et al., 1986). Expression of these new Tn*5 lac* reporter genes was examined in *asg* mutant strains to determine if the *asg* defect altered their expression patterns (Kuspa et al., 1986). Assuming that the developmental pathway is linear and unbranched, the prediction is that those genes normally expressed after the *asg* block would not be expressed in the *asg* mutant background. Twenty-one developmentally-expressed reporter genes were separated into two groups following examination of their expression patterns in wild-type and *asg* mutant backgrounds. Three genes termed "A-signal-independent" have the same expression pattern in the wild-type and *asg* mutant backgrounds. These genes increase in expression between 0 and 3 h of development. The expression of the other 18 genes, termed "A-signal-dependent", is reduced or abolished in the *asg* mutant background. These genes increase in expression only after 1.5 h of development. These data indicate that the *asg* block occurs within the first or second hour of development. Thus, A signal functions very early in *M. xanthus* development.

At this point, the stage was set to test predictions of the original cell-cell signaling hypothesis: If the *asg* mutants are defective in release of an extracellular signal, and the absence of that signal results in failure to express "A-signal-

dependent" Tn*5 lac* insertion genes, then expression of these genes in an *asg* mutant should be restored by addition of wild-type cells. Further, if the extracellular signal is indeed released by wild-type cells, then it should be possible to recover the activity from suspensions of developing wild-type cells. This "conditioned medium," when added to *asg* mutant cells, should result in restored expression of the A-signal-dependent genes. All of these predictions were found to hold true, and in the process of testing them, the A-signal (also referred to as A factor) bioassay was developed (Kuspa et al., 1986).

A bioassay for A signal

The "detector" in the bioassay for A-signal activity is an *asg* mutant strain that contains the Tn*5lac* insertion Ω4521. Expression of the *4521* gene initiates at 1.5 hr of development in wild-type cells, and is abolished in *asg* mutant cells. A-signal activity is detected by measuring the increase in β-galactosidase activity that results when A signal is supplied to the starving detector cells (Kuspa et al., 1986). The detector cells are suspended in buffer and placed in multi-well tissue culture plates. Cells or extracts are added to the wells to test for the presence of A-signal activity. The plates are incubated for 20 hr, after which cells are removed and assayed for β-galactosidase activity. Although the cells in this bioassay do not form fruiting bodies because they are suspended in liquid rather than on a solid surface, early steps in development have been shown to occur under these conditions (Downard and Zusman, 1985, Kuspa et al., 1986). The A-signal bioassay shows a linear response to the addition of A signal, and thus can be used for quantitation of A-signal activity (Kuspa et al., 1986).

Purification of the A signal: proteases and amino acids

With the development of the A-signal bioassay, the tools for identification of the A-signal molecule(s) were in place. Kuspa *et al.* (Kuspa et al., 1986) showed that A-signal activity is released from wild-type cells beginning an hour or so after cells are placed under starvation conditions-- including starvation in shaken suspension. Removing the cells from such a suspension leaves behind a translucent supernatant, which is a convenient starting material for A-signal purification. Early in the A-signal purification, it was noted that some of the activity is rapidly inactivated by heating, while the remaining activity is stable to heating at 100°C for 10 minutes. The heat-stable activity was found to pass through dialysis tubing with a 3-kDa molecular size cut-off and to be included in gel filtration columns with molecular exclusion limits of 2 kDa. The heat-labile activity was found to be non-dialyzable, and seemed to co-purify with proteolytic activity. Therefore, it was concluded that at least two physically different types of A-signal activity exist (Plamann et al., 1992).

Proteolytic activities were measured throughout the A-signal purification, and it was noted that proteolytic activity consistently co-purified with A-signal activity. Plamann *et al.* (Plamann et al., 1992) found that developing wild-type cells release at least two different proteins that possess both proteolytic and A-signal activities. One of these proteases is a 27 kDa protein with a trypsin-like specificity. The second protease has a molecular mass of approximately 10 kDa and shows a pattern of substrate specificity that differs from that of the 27 kDa protein. Because two proteases with seemingly different substrate specificities were found to possess A-signal activity, it seemed possible that common laboratory proteases might have A-signal activity. Indeed, it was found that pronase, proteinase K, papain, trypsin and chymotrypsin all have heat-labile A-signal activity (Plamann et al., 1992). Furthermore, it was found that pronase, and to a lesser extent trypsin, rescue fruiting body formation in the *asg* mutants (Kuspa et al., 1992). Thus, it appeared

that heat-labile A signal is a mixture of proteases. Because the cell envelope is remodeled as the rod-shaped cell differentiates to form the ovoid myxospore, it is reasonable to assume that proteins not needed by the myxospore serve as a substrate for the A-signal proteases. The purification of heat-stable A signal and its identification as amino acids and peptides supports the hypothesis that the peptides and amino acids released as a consequence of extracellular proteolysis act as the true A signal.

Fifteen different amino acids have A-signal activity (Kuspa et al., 1992). Any one of these amino acids, when added to *asg* mutant cells in the A-signal assay, restores Tn*5* *lac* Ω4521 β-galactosidase activity to a near normal level. The amino acids with the highest A-signal activities are tyrosine, proline, phenylalanine, tryptophan, leucine, isoleucine, and alanine; these amino acids can account for approximately half of the heat-stable A-signal activity in the cell-free supernatants. The threshold concentrations for these amino acids is approximately 10 μM; below this concentration, *4521* expression is not rescued in an *asg* mutant. Small peptides also have heat-stable A-signal activity. Their activities can be roughly approximated by adding up the activity of the individual amino acids that comprise the peptides. Kuspa *et al.* (Kuspa et al., 1992) suggest that the half of heat-stable A signal that is not accounted for by individual amino acids may be comprised of small peptides with fewer than 100 amino acid residues.

There is considerable evidence to support the hypothesis that A signal is generated by extracellular proteases as they degrade proteins to peptides and finally to amino acids, which are the primary A signal. First, cell-free conditioned buffer autolysed in vitro, resulting in a 1.7-fold increase in the levels of free amino acids. Heat-stable A-signal activity also increased by 1.7-fold in these experiments. In addition, developing wild-type cells released amino acids and peptides at the same time that A-signal activity appeared in conditioned buffer, and it has been

estimated that the amount of extracellular amino acids generated by wild-type cells during the first 12 hours of development is sufficient to account for the observed level of *4521* expression (Kuspa et al., 1992). Finally, two proteases with differing substrate specificities were isolated from buffer conditioned by developing wild-type cells, and all commercially available proteases that were tested have A-signal activity (Plamann et al., 1992).

A signal as a cell density signal

The logic behind the hypothesis that A signal is a cell-density signal for development is as follows: First, *4521* expression gene requires a high cell density (Kuspa et al., 1992). Second, *4521* is not expressed in an *asg* mutant (Kuspa et al., 1986). Third, *4521* expression in an *asg* mutant is rescued by addition of amino acids (Kuspa et al., 1992, Kuspa et al., 1992). Finally, the concentration of extracellular amino acids is proportional to the concentration of cells undergoing early development (Kuspa et al., 1992). If the above hypothesis is correct, then *4521* expression at low density should be rescued by the addition of amino acids. This is precisely what was observed (Kuspa et al., 1992). Furthermore, it was found that *asg* mutants, which release 5 to 10% of the normal level of A signal, express *4521* and sporulate when their cell density is raised 10- to 20-fold above the normal density required for development.

Assuming extracellular amino acids induce *4521* expression and development, what prevents these processes from being triggered during growth when the extracellular concentration of amino acids is high? It is likely that the mechanisms for sensing and responding to extracellular A signal are operational only when the cells are starving. Genetic suppression analysis detailed below has begun to address this issue.

Why does *M. xanthus* use amino acids as a cell-density signal? Perhaps it is a question of metabolic economics. It makes "economic sense" that extracellular and periplasmic proteins dispensable for fruiting body formation and spore production are recycled during development. Instead of taking on the expense of producing an alternative cell density signal during early development, *M. xanthus* may have evolved a mechanism to use the extracellular amino acids as both a carbon source and as a signal for development.

Three *asg* loci

In the original screen for cell-cell signaling mutants, Hagen *et al.* (Hagen et al., 1978) identified 18 *asg* (group A) mutants, which represents about one-third of the total number of extracellularly complementable mutants isolated. The *asg* mutations map to three unlinked loci: *asgA*, *asgB*, and *asgC* (Kuspa and Kaiser, 1989). The three types of *asg* mutants share a number of phenotypic characteristics in addition to their defects in A-signal generation and fruiting body formation. For example, wild-type cells exhibit tan-to-yellow and yellow-to-tan phase variation, while *asg mutants* are permanently tan. In addition, the *asg* mutants have less tendency to form clumps in liquid culture, and they release less extracellular protein. All of these phenotypes are related in some way to defects in the cell surface or the extracellular matrix, which led to the suggestion that the *asg* mutations in some way alter export (Kuspa and Kaiser, 1989), including export of the proteases that generate A signal.

DNA sequence analyses of the *asgA*, *asgB*, and *asgC* genes resulted in significant advances in our understanding of the cellular roles of these genes (Davis et al., 1995, Plamann et al., 1994, Plamann et al., 1995). The *asgA* gene encodes a histidine protein kinase that contains domains that are highly conserved among sensor kinases and response regulators of two-component signal transduction

systems. These are now widely recognized for their roles in sensing and responding to environmental signals in bacteria (Plamann et al., 1995), and more recently have been identified in eukaryotic systems (Appleby et al., 1996). The paradigm two-component system consists of a membrane-bound sensor that detects an environmental stimulus, and a cytoplasmic response regulator protein that alters gene expression in response to signals from the sensor. Commonly, each of the two proteins contain two domains. The sensor contains an input domain that interacts with the environmental signal, and a second domain that catalyzes autophosphorylation (histidine protein kinase domain). Typically, the response regulator contains a receiver domain that removes the phosphate from the sensor and attaches it to an aspartate, and a DNA binding domain that regulates gene expression (Parkinson, 1993, Stock et al., 1989). AsgA is an unusual regulatory protein in that it consists entirely of a receiver domain followed by a histidine protein kinase domain, and appears to lack the membrane-spanning, hydrophobic regions that characterize most of the sensors (Plamann et al., 1995). AsgA protein has been purified and shown to have autokinase activity (Li and Plamann, 1996).

As described above, in the paradigm two-component signal transduction system, the histidine protein kinase domain of the sensor and the receiver domain of the response regulator are located on separate polypeptides along with their associated input and output domains, respectively. However, many, if not most "two-component" signal transduction systems exhibit variations on this theme. Some systems are composed of three or more proteins, and the different modules may have been separated from one another or rearranged to give rise to alternative signal transduction circuits (Parkinson and Kofoid, 1992). In *Bacillus subtilis*, a phosphorelay containing proteins homologous to response regulators and histidine protein kinases (as well as other proteins) controls the initiation of sporulation (Burbulys et al., 1991). In this phosphorelay, phosphoryl groups are transferred

from a histidine, to an aspartate, to a histidine, to an aspartate. Similar or related His-Asp-His-Asp phosphorelays have been reported, and it has been proposed that such a relay could function to ensure that the signal is of sufficient intensity or duration before embarking on a "costly" response such as sporulation (Appleby et al., 1996). Furthermore, a relay provides additional points at which multiple signals may influence the flow of information and affect the decision to sporulate (Burbulys et al., 1991, Grossman, 1991, Ireton et al., 1993). Perhaps the role of AsgA in A-signal generation is to function within a phosphorelay that begins with a starvation-sensing autokinase, and ends with a transcriptional regulator that affects genes required for A-signal generation (see Figure 1 and text below).

The *asgB* gene encodes a 163 amino acid polypeptide with a potential helix-turn-helix (HTH) DNA-binding motif near its C-terminus (Plamann et al., 1994). This predicted HTH is highly similar to the HTH found in region 4 of the major sigma factors. Sigma factor region 4 is one of the two most highly conserved regions among the sigma factors and is characterized by its location at the C-terminus and a conserved HTH. There is considerable genetic and biochemical evidence to suggest that this HTH directly contacts the -35 region (TTGACA) of promoter sequences (Dombroski et al., 1992, Gardella et al., 1989, Lonetto et al., 1992, Margolis et al., 1991, Siegele et al., 1989). AsgB does not contain sequences that are similar to conserved sigma factor regions 1, 2 or 3; therefore, it is unlikely that AsgB functions as a sigma factor (Plamann et al., 1994). A more likely scenario is that AsgB is a transcription factor that recognizes a DNA sequence closely related to the -35 hexamer. If AsgB makes contacts similar to those made by sigma and binds to -35 regions in *M. xanthus*, it could repress transcription by competing with the major form of RNA polymerase holoenzyme for specific binding to promoters. One simple model, which takes into account that *asgB* appears to be essential for growth (Plamann et al., 1994), is that AsgB represses transcription of an early class

of developmental genes during growth. In this model, it is assumed that transcription of these early developmental genes is lethal in growing cells, and that the mutant containing the *asgB480* point mutation is defective in developmental derepression. Alternative models are that AsgB is an activator or a repressor that binds to nonpromoter sequences, or that AsgB is part of a multicomponent sigma factor in which the AsgB subunit provides specificity for the -35 region, while other proteins allow interaction with RNA polymerase or other promoter sequences.

The *asgC* gene has been localized to a region that contains genes homologous to *rpsU*, *dnaG*, and *rpoD* of the *Escherichia coli* macromolecular synthesis operon (Davis et al., 1995). These genes encode proteins that are required for the initiation of protein, DNA, and RNA synthesis, respectively (Versalovic et al., 1993). The *asgC767* mutation was identified as two consecutive base substitutions resulting in a glutamate to lysine substitution within the *rpoD* homolog (known as *sigA*), which encodes the major sigma factor in *M. xanthus*. This amino acid substitution is at position 598 within sigma factor conserved region 3 (Davis et al., 1995).

One possible explanation for the A-signaling defect observed in the *asgC* (*sigA*) mutant strain is that the mutant sigma subunit fails to interact productively with a transcriptional regulator that affects A-signal generation. Mutations that affect interactions between RNA polymerase and transcription factors have been identified in the genes encoding the alpha and sigma subunits of RNA polymerase (Ishihama, 1993). *E. coli* sigma mutations that affect interactions with the transcription factors PhoB (Makino et al., 1993), CRP (at the P1*gal* promoter) (Kolb et al., 1993) AraC (Hu and Gross, 1985), and the λ cI repressor (Kuldell and Hochschild, 1994, Li et al., 1994) have been localized to region 4. Kumar et al. (Kumar et al., 1994) suggest, from data provided through a deletional analysis of the C-terminal portions of *rpoD*, that a region extending from at least region 3.2 to upstream of region 4.2 may be involved in association with transcription factors.

More recently, Bramucci et al. (Bramucci et al., 1995) identified a mutation in a *B. subtilis* sigma factor gene (*spo0H*) that suppresses the transcriptional defects of a mutant form of the transcription factor Spo0A (Spo0A9V). This *spo0H* mutation is located between the sequences encoding regions 2 and 3 and is proposed to allow SpoOA9V to interact with the mutant sigma-H, restoring transcriptional activation. Similarly, the A-signaling defect observed in the *asgC*(*sigA*) mutant strain may be caused by a failure of the mutant sigma subunit to interact functionally with a transcriptional regulator (AsgB?) necessary for A-signal production. An alternative hypothesis is that the mutant sigma factor has a greater affinity for core polymerase, preventing alternative sigma factors necessary for A-signal generation from gaining access to core.

Recently, Hernandez and Cashel (Hernandez and Cashel, 1995) identified *E. coli rpoD* mutations that suppress the defects of a strain unable to synthesize ppGpp. The phenotypes of the *E. coli* mutants suggest that they are hypersensitive to ppGpp, or that their RNA polymerase acts as if ppGpp is already present. Interestingly, the mutations result in substitutions within conserved region 3.1 of *E. coli* RpoD, immediately adjacent to the site of the substitutions in the *M. xanthus asgC* (*sigA*) mutant. It may be that the *M. xanthus asgC* mutant, rather than being hypersensitive to ppGpp, is insensitive to ppGpp, and therefore, is unable to alter gene expression in response to starvation. This hypothesis fits well with the phenotype of the *asgC* mutant, and does not exclude the transcriptional regulator/sigma factor/interaction hypothesis explained above, because RNA polymerase may respond indirectly to ppGpp, or to ppGpp that is associated with a regulatory protein.

A model for the A-signal-generating pathway

Figure 1 illustrates one of many plausible models for the role of the *asg* gene products in A-signal generation. Given that the *asg* mutants have very similar phenotypes and the *asg* genes encode proteins similar to ones with known regulatory functions, we hypothesize that the *asg* gene products function together in a signal transduction pathway that is required for generation of extracellular A signal. In the figure, AsgA (a histidine protein kinase) is shown interacting with an unknown starvation sensor, which may be another histidine protein kinase, a serine/threonine kinase, or a small-molecule phosphodonor. This interaction results in activation of the AsgA autokinase, and AsgA is converted to AsgA-phosphate. The phosphoryl group is then transferred to an unidentified protein or through a series of proteins, and finally to AsgB (a putative transcription factor). In this model, AsgB acts as a repressor of early developmental genes when it is unphosphorylated, and/or as an activator of these genes when phosphorylated. Expression of the genes required for A-signal generation requires the wild-type major sigma factor (SigA), perhaps for interaction with AsgB and/or ppGpp. Expression of these genes, which may include genes encoding proteases or secretory machinery, results in release of extracellular proteases that generate A signal. Finally, sensing and transduction of A signal leads to the expression of A-signal-dependent genes such as *4521*. It is easy to imagine several variations of this model. For example, the regulator at the downstream end of the pathway may be a transcriptional activator whose production is regulated by AsgB, or AsgA may function downstream rather than upstream of AsgB.

The anatomy of an A-signal-dependent gene: *4521*

One class of *asg*-dependent developmentally expressed genes appears to be the primary target of A signal (Bowden and Kaplan, 1996). Expression of these genes, identified by the Tn*5* *lac* insertions Ω4442, Ω4457, Ω4494, and Ω4521, begins

between 1 to 3 h after the initiation of development and is cell-density-dependent. In wild-type cells, their expression levels are low at low cell densities and rise dramatically as the cell density is increased above 5 x 10^8 cells per ml. In cells at low density, expression of these genes can be rescued to near maximum levels per cell if A signal is added.

The best characterized member of this class is *4521*. Its expression pattern has been analyzed directly by measuring RNA accumulation (Kaplan et al., 1991), and indirectly by measuring the β-galactosidase activity of strains containing the Ω4521 Tn*5 lac* fusion (Kaplan et al., 1991, Kuspa et al., 1986, Plamann et al., 1995). Expression of *4521* requires independent input from both starvation and A signal (Kaplan et al., 1991), and occurs only if the cells are starving and at a high cell density or starving and exposed to extracellular A signal. In common with the expression of the other genes in this class, *4521* expression can be restored to starving *asg* mutants or starving low density cells by the addition of exogenous A signal (Kuspa et al., 1986). The expression of *4521* can also be restored by the presence of *asg* suppressor mutations (Kaplan et al., 1991), designated *sas*.

Studies of the *cis*-acting elements controlling *4521* expression have identified the *4521* promoter as a member of the sigma54 family (Keseler and Kaiser, 1995). Keseler and Kaiser (Keseler and Kaiser, 1995) identified sequences upstream of the *4521* transcription start site (TSS) that resemble but do not perfectly match those present in the sigma54 consensus promoter. The conserved GC dinucleotides normally present at -12 are located at -14/-15 upstream of the *4521* TSS. The GG dinucleotide normally present at -24 is proposed to correspond with the AG dinucleotide at -26/-27. The *4521* promoter does include the sigma54 consensus pyrimidine-purine-pyrimidine -purine pattern at -24, a 10-base pair separation of the -12 and -24 dinucleotides, and consensus TT at -16. A mutational analysis of the conserved residues indicates that altering bases within the conserved regions

greatly reduces *4521 in vivo* expression. In addition, changes which alter the spacing between the two conserved regions abolishes promoter activity.

A 5'-deletion analysis of the DNA upstream of the *4521* TSS determined that, at most, 146 bp upstream of the *4521* TSS is required for wild-type *4521* expression during growth and development (Gulati et al., 1995). This result is consistent with the fact that all other known sigma54 promoters require binding of an activator protein to sites located upstream of the promoter prior to open complex formation (Kustu et al., 1989). The *Klebsiella pneumoniae* NtrC protein, which is involved in nitrogen assimilation, is the best characterized sigma54-dependent transcriptional activator. NtrC is the response regulator in a two component signal transduction system in which NtrB is the sensor histidine kinase. To determine if the *M. xanthus* chromosome might encode a sigma54-dependent transcriptional activator, Kaufman and Nixon (Kaufman and Nixon, 1996) used degenerate oligonucleotides and PCR to amplify gene fragments from the *M. xanthus* chromosome that encode the conserved catalytic domain of sigma54-dependent transcriptional activators. They identified 14 different fragments, suggesting that *M. xanthus* appears to contain many sigma54-dependent transcriptional activators. Among these activators are the previously identified PilR which regulates *M. xanthus pilA* (the pilin structural gene) (Wu and Kaiser, 1995), and SasR, a putative activator of *4521* expression (Gorski and Kaiser, 1997, Yang and Kaplan, 1997).

Consistent with the possibility that the *4521* promoter is a member of the sigma54 family, Keseler and Kaiser (Keseler and Kaiser, 1995) have identified and characterized a *M. xanthus* gene, *rpoN*, predicted to encode a sigma54 homolog. This gene was identified by cross-hybridization to the *Caulobacter crescentus rpoN* gene. Interestingly, attempts to generate a *rpoN* null mutation were not successful, suggesting that this sigma54 is essential for *M. xanthus* growth. This represents the first example of an alternative sigma factor that is vital to cell growth.

Unfortunately, the inability to generate an *rpoN* null makes it difficult to test the sigma54-dependent expression of *4521* and other genes in this class.

The chromosomal *M. xanthus* DNA into which the Ω4521 Tn*5 lac* inserted was cloned, and the nucleotide sequence of a 1.7 kb region has been determined. The sequence analysis reveals one open reading frame (ORF) of 1311 bp that codes for a predicted polypeptide that shares 24 to 33% identity with members of the serpin family of serine proteinase inhibitors (Gulati et al., 1997). Members of this family include human plasma proteins such as antithrombin and antitrypsin, and human tissue proteins such as plasminogen activator inhibitor (Potempa et al., 1994). To our knowledge this is the first bacterial member of the serpin family.

One model suggested by the *4521* sequence analysis is that the *M. xanthus* serpin homologue is a proteinase inhibitor that feedback inhibits a serine proteinase, thus decreasing the concentration of extracellular A signal. The pattern of A-signal activity reveals that extracellular A signal increases at about 1 h after development is initiated and begins to decrease at about 3 h of development (Kuspa et al., 1986). This decrease in A-signal activity could be a result of a number of overlapping phenomena such as a decrease in the release of proteinases, a reduction in the amount of protein substrate, and the inhibition of the proteinases. Interestingly, the Ω4521 Tn*5 lac* insertion does not have an obvious effect on growth or development, suggesting that either *4521* is not critical to growth and development, or that other genes serve similar functions.

Genetic suppression analysis

Dissecting the circuitry that connects extracellular A signal to its responsive genes is an important aspect of the analysis of the A signaling system. The A-signaling system appeared to be an ideal system for genetic analyses; the phenotypic characteristics of mutants could be predicted, and a few of the components were known. In addition, the genetic tools for such an analysis in *M. xanthus* were available. It was possible to transfer genes between *M. xanthus* strains and between *E. coli* and *M. xanthus*, allowing mutations to be mapped and tests of complementation, dominance, and epistasis to be performed (Gill and Shimkets, 1993). A suppressor screen could therefore be used to identify A-signal transducers.

Early studies indicated that the control of *4521* expression by extracellular A signal is at the transcriptional level (Kaplan et al., 1991). This suggested that the A-signal transducers are regulators of *4521* transcription. A genetic screen was developed to identify these elements based on their ability to suppress the *asgB480* mutation. This scheme was expected to identify mutations that permitted *4521* expression in the absence of A signal. The mutant phenotypes could result from loss-of-function mutations of negative regulators or gain-of-function mutations of positive regulators.

Specifically, *asgB480* mutants containing the Tn*5 lac* Ω4521 fusion strain were UV mutagenized and plated on nutrient agar containing X-Gal (Kaplan et al., 1991). Those mutants that expressed *4521* generated blue colonies among a lawn of tan parents that did not express *4521*. Six suppressor mutants (*sasB5*, *7*, *14*, *15*, *16*, *17*) were isolated that bypass both the starvation and A-signal requirements for *4521* expression. In these mutants *4521* is expressed during growth and development in the absence of A signal.

Interestingly, the six suppressor mutations map to the same locus, *sasB* (Kaplan et al., 1991), which encodes a number of transducer-like proteins (Fig. 2). One of the suppressor mutations, *sasB7* maps to the *sasS* gene. The sequence of the wild-type *sasS* gene predicts that it encodes a transmembrane histidine kinase sensor typical of the type found in two-component signal transduction systems (Yang and Kaplan, 1997). The N-terminus of SasS is predicted to contain two transmembrane domains. These two transmembrane domains are similar to TM1 and TM2 of the *E. coli* chemotaxis receptors (MCPs) (25 to 40 and 20 to 25% identical) (Bolinger et al., 1984, Boyd et al., 1983, Krikos et al., 1983). In addition, the whole N-terminal input domain (amino acids 1 to 190) of SasS has limited homology (about 21% identity) to all the *E. coli* MCPs (Bolinger et al., 1984, Boyd et al., 1983, Krikos et al., 1983). The C terminus of SasS contains all of the conserved residues typically found in sensor histidine protein kinases.

To study the function of *sasS* in response to A signal, null mutants of *sasS* were generated and characterized. Surprisingly, the null mutants had an opposite effect on *4521* expression, relative to the *sasB7* mutation which identified the gene. The *sasS* null mutants express *4521* at a basal level. These data indicate that the wild-type *sasS* gene product functions as a positive regulator of *4521* expression and that the *sasB7* is a gain-of-function allele. Further analysis of the *sasS* null mutants revealed that the mutants generate normal A-signal levels, but they do not respond to exogenous A signal. In addition the *sasS* null mutants form defective fruiting bodies and sporulate less efficiently. These data indicate that the wild-type *sasS* gene product functions as a positive regulator of *4521* expression and participates in *M. xanthus* development. Most importantly, SasS appears to be a key element in the transduction of starvation and extracellular A signal. It is predicted to function by controlling the phosphorylation level of a downstream response regulator that affects expression of genes such as *4521*.

Subsequent suppressor analysis has identified, 3 kb downstream of *sasS*, a putative cognate response regulator for SasS (Yang and Kaplan, 1997). This *sasR* gene is predicted to encode a sigma54-dependent transcriptional activator and, similar to NtrC, contains receiver, catalytic and DNA-binding domains. This gene was among those identified by Kaufman and Nixon in their PCR amplification (Kaufman and Nixon, 1996). The phenotype of the *sasR* null mutants indicate that SasR, like SasS, functions as a positive regulator of *4521* expression and is important for normal fruiting body formation and sporulation (Gorski and Kaiser, 1997, Yang and Kaplan, 1997).

The five other original suppressor mutations map to the *sasB* gene which is located directly downstream of *sasR* (Xu et al., 1997). The *sasB* gene product has no homologues in the databases. Its distinguishing characteristics are in its N-terminus, and include an amino acid hydrophobic region (amino acids 9 to 35) and a leucine zipper motif (amino acids 64 to 85). The phenotypes of the *sasB* null mutants are identical to the original point mutations; that is the null mutants bypass both the starvation and A-signal requirements for *4521* expression. Expression of *4521* is very high during growth and development, yet increases still further at about 2 h into development. These data indicate that SasB functions as a negative regulator of *4521* expression. The point mutations appear to be a result of two independent mutations. The *sasB16* mutation generates a nonsense codon at amino acid 47. Mutations *sasB5*, *-14*, *-15*, and *-17* are identical and cause a threonine to proline substitution at amino acid 280. In addition, the *sasB* null mutants form defective fruiting bodies and sporulate at about 10% of the wild-type level, indicating that the wild-type SasB is necessary for normal fruiting body formation and sporulation.

Once SasS, SasR, and SasB were identified it was possible to examine their relation to each other in transducing cell density information in response to

extracellular A signal. The order of function of these gene products was addressed genetically through epistasis analysis. Epistasis analysis is performed by placing two mutations that generate different phenotypes into the same strain. The outcome phenotype represents the function that is farther downstream in a signal transduction pathway (Parkinson, 1995). The results of such pairwise analysis with SasB, SasS and SasR indicate that, assuming that they are in the same pathway, they function in the order listed (Xu et al., 1997).

Models that postulate a mechanism of A-signal transduction based on the data described above predict that SasS is stably phosphorylated when extracellular A signal is above its minimum threshold concentration, and that this phosphate is transferred to SasR, which activates transcription from the *4521* promoter. It is likely that SasS senses A signal by direct binding of amino acids or through interactions with amino acid-binding proteins. In either case, SasS would determine whether the concentration of A-signal amino acids in the periplasmic space is above 10 μM. This type of mechanism would be typical of interactions between a ligand and its binding protein. This possibility is particularly intriguing when it is considered that A signal is a set of amino acids, and that the predicted SasS N-terminal input domain structure is similar to that of the MCP chemoreceptors, many of which directly sense amino acids.

Two possible models can account for the activity of SasB. The model we favor is based on the idea that SasB, SasS, and SasR function in a linear pathway (Fig. 3). In this model SasB inhibits the activity of the SasS sensor during growth resulting in basal *4521* expression. Then as a consequence of starvation, SasB is inactivated allowing SasS to be stably phosphorylated when the A-signal concentration surpasses its threshold leading to *4521* expression. However, it is also possible that SasB is in a separate pathway from SasS, and its negative activity impacts the *4521*

promoter more directly. This latter possibility seems unlikely based on the high *4521* expression in strains containing the *sasS* gain-of-function mutation.

An interesting corollary of the first model may explain why *4521* expression is low during growth even though the periplasmic A-signal amino acid concentrations should be above the 10 μM threshold. The model predicts that SasB inhibits the ability of the SasS sensor to serve as a source of phosphate for SasR during growth. SasB might inhibit the SasS autokinase activity or could function as a phosphatase. Considering the adaptation response of the chemoreceptors, this inhibition may be viewed as desensitizing SasS to the high amino acid concentration during growth.

Future directions

The numerous signaling pathways involved early in M. xanthus fruiting body development indicate that many aspects of the cells' environment are assessed when they are poised at the transition between growth and development. Our current knowledge suggests that what was initially viewed as a simple linear set of signaling pathways -- starvation sensing, generation of A signal, and reception and response to A signal -- is more likely to be an interconnected network. The challenge for the future will be to understand these complex interconnections.

The application of genetic methods to identify the components of these systems, followed by biochemical studies to characterize their mechanisms of action, is an approach that has proved to be successful in the past and will be continued into the future. Specifically, for the A-signal-generating pathway, a genetic suppression analysis is currently in use to identify genes whose products interact with the known *asg* gene products. Further understanding of the A-signal-response pathway requires biochemical approaches to examine the activities of the putative transducers SasS, SasR and SasB. These experiments will address the specificity

for the subset of amino acids that comprise A signal and the concentration threshold for activity. Finally, a comprehensive analysis of A-signal generation and response requires that the scope of the investigation be broadened to include *asg*-dependent genes other than *4521*, some of which may be required for fruiting body formation.

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Yang, C. and H. B. Kaplan. Unpublished data.

Figure 1. A model of the A-signal generating pathway. AsgA is a histidine protein kinase that is predicted to function within a phosphorelay that senses starvation and regulates the expression of genes required for A-signal generation. Unknown proteins (Asg?) are shown interacting upstream and downstream of AsgA in this phosphorelay. AsgB is predicted to be a transcriptional regulator. SigA (AsgC) is the major sigma factor in *M. xanthus*. See text for a more detailed description.

Figure 2. Physical map of the *sasB* locus. Genetic and molecular analysis of wild-type and mutated *sasB* alleles were used to develop a map of the *sasB* locus. The line represents the *M. xanthus* chromosome. The horizontal arrows indicate genetically determined individual transcription units and their direction of transcription. The vertical arrows indicate the location of point mutations that identified the region. The boxes indicate the position of ORFs. The wedges indicate the phenotypes of null mutations in the ORFs: open wedges represent null mutants that express the *4521* reporter gene at basal levels, closed wedges represent null mutants that express the *4521* reporter gene at high levels during growth and development.

Figure 3. A model of the A-signal-response pathway. An individual *M. xanthus* cell is represented during early development to illustrate the relationship between the elements and the signaling molecules controlling *4521* gene expression. Arrows indicate positive biochemical or genetic functions and lines ending in bars indicate negative biochemical or genetic functions. Genes enclosed in white shapes are currently understudy in our laboratories. RpoN + RNAP represents *M. xanthus* RNA polymerase sigma54 holoenzyme. The bent arrow indicates transcription of the *4521* gene. In this model, the SasB inhibitor is inactivated in starving cells. By 1 - 2 h after starvation at high cell density, the extracellular A-signal concentration

surpasses the activation threshold of 10 μM, thereby activating the autokinase activity of the SasS sensor. This phosphate is then transferred to the SasR activator resulting in *4521* transcription. The expression of *4521* appears also to be controlled by an undetermined starvation-dependent and A-signal-independent mechanism indicated by the thin line and question mark.

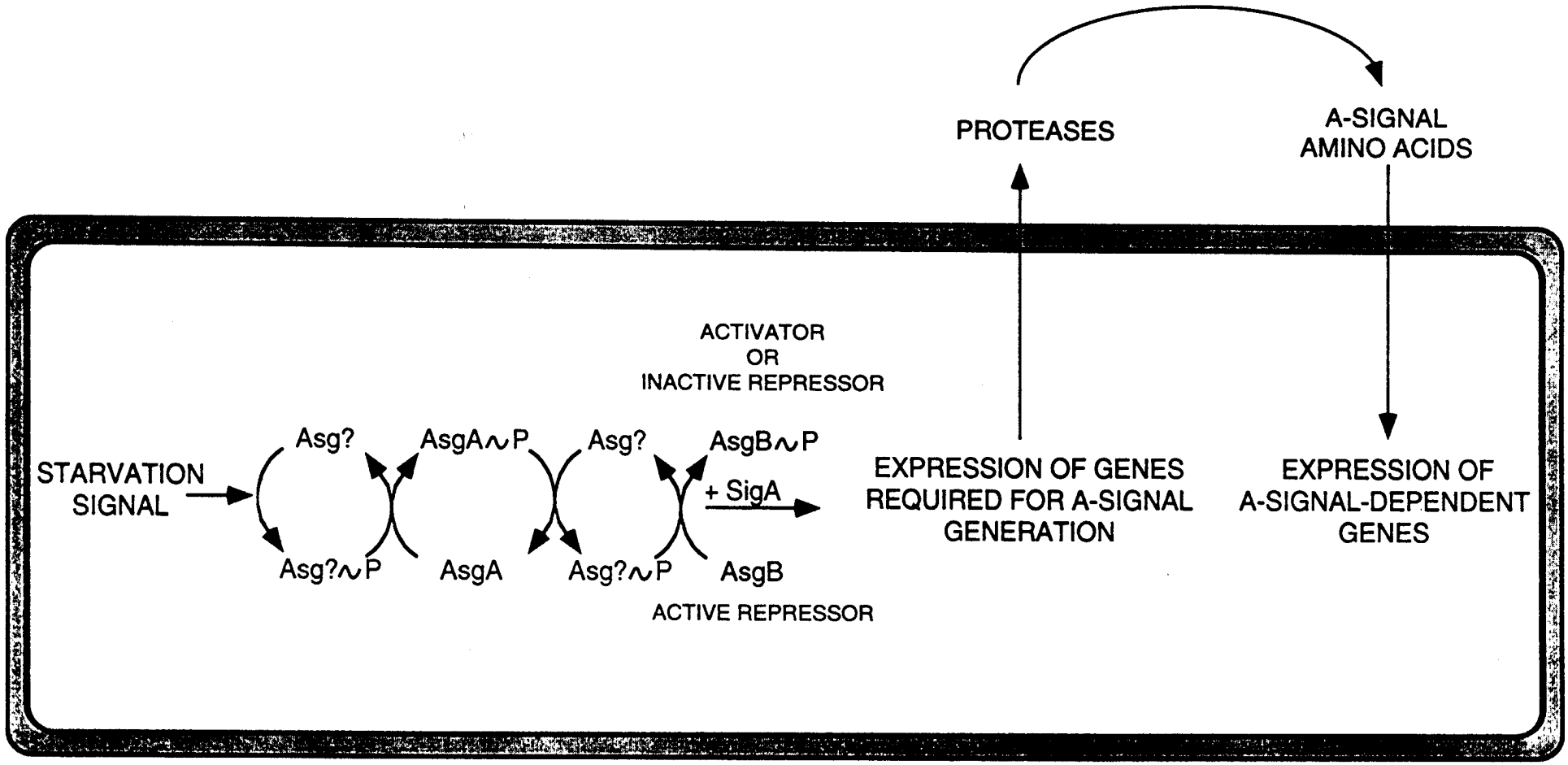
PROTEASES
A-SIGNAL
AMINO ACIDS
ACTIVATOR
OR
INACTIVE REPRESSOR
STARVATION
SIGNAL
Asg?
AsgA∼P
Asg?
AsgB∼P
+ SigA
Asg?∼P
AsgA
Asg?∼P
AsgB
ACTIVE REPRESSOR
EXPRESSION OF GENES
REQUIRED FOR A-SIGNAL
GENERATION
EXPRESSION OF
A-SIGNAL-DEPENDENT
GENES

Figure 2, Plamann & Kaplan

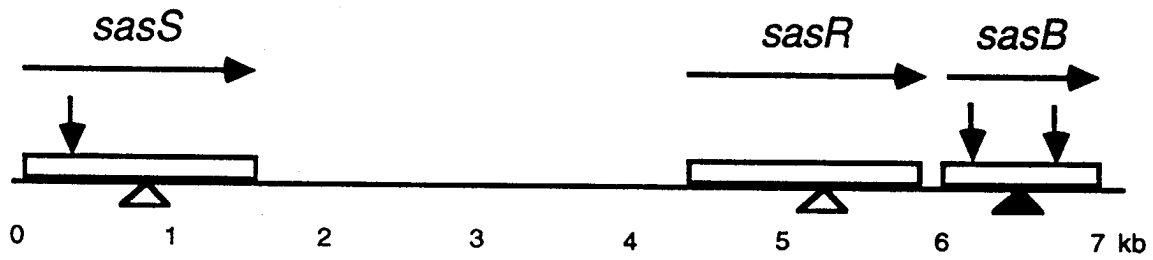

Figure 5, Transcription of Regulation